THE CALCULATION OF TRUE DIPOLE MOMENTS FROM SOLUTIONS IN POLAR SOLVENTS

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*Summary*

The theory presented in an earlier paper is here developed as a method for the evaluation of true dipole moments from data obtained from solutions in polar solvents. As illustrations, the new method is applied to values relating to solutions of several substances in chloroform, chlorobenzene, and nitrobenzene. The results, considering the nature of the problem, are satisfactory. The accuracy is greatest when the solute is highly polar and the dielectric constant of the solvent small. Aqueous solutions of pyridine and of two amino acids are also considered. In the latter cases especially, the predicted dipole moments agree most favourably with anticipated values obtained for the zwitterions by multiplying the electronic charge by the expected charge separation.

## I. INTRODUCTION

The several possible conditions under which dipole moments can be measured via the Debye theory, arranged in order of increasing complexity, are :

(i) The vapour state, when, at low pressures, negligible molecular interaction occurs.

(ii) A dilute solution in a non-polar solvent, in which case the effect of the field of the polar molecule on its " neutral " surroundings must be considered ; the " reaction " field thus becomes appreciable.

(iii) The pure liquid, when every molecule is a " source " of polarization, but all are identical.

(iv) A solution containing more than one species of polar molecule.

The theory presented in an earlier paper (Buckingham 1953), being quite general, should be capable of application to mixtures of polar substances. This aspect is now developed in Section II, and expressions derived there are tested in Section III by applying them to values relating to solutions in several polar solvents, and in Section IV, by substituting in them data drawn from solutions of pyridine and of two dipolar-ionic structures, namely, glycine and β-alanine, in water. The properties of the solute molecule which are required are simply its " internal " refractive index and its approximate shape. These factors, together with the dielectric constant and density of a solution of known concentration, are sufficient to enable an estimate to be made of the true, or " gas " dipole moment of the molecules of the solute.

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Since there has in the past been no method by which reliable dipole moments may be calculated from observations made on solutions in solvents of high dielectric constant (for example, water) the formulae now presented could be of some assistance in the determination of structures of complex molecules—such as proteins, which are not soluble in non-polar media.

## II. Derivation of the Equations

The static dielectric constant $\varepsilon$, of a solution containing $N_i$ molecules of the $i$th species in unit volume, was shown by Buckingham (1953) to be related to molecular properties by the equation

$$\frac{(2\varepsilon+1)(\varepsilon-n^2)}{3(2\varepsilon+n^2)}=\frac{4\pi}{9kT}\Sigma N_i(\mu_{0i})^2 f_i g_i, \qquad (1)$$

where $n^2$ is the "effective" refractive index of the solution, and where the moment of a spherical specimen of the liquid, containing at the centre a molecule of "gas" moment $\mu_{0i}$, is $f_i\mu_{0i}$, in the absence of an applied field. The factor $g_i$ is related to the energy $w$, of a molecule of moment $\mu_{0i}$ when a uniform field of strength $\mathbf{E}$ exists in the solution, by the expression

$$w=-g_i\mu_{0i}E\cos\theta,$$

$\theta$ being the angle between $\mathbf{E}$ and the axis of the dipole. Both $f_i$ and $g_i$ depend on $\varepsilon$ and on constants relevant to the particular molecule concerned.

Now

$$N_i=x_iN, \qquad (2)$$

where $x_i$ is the mole fraction of the $i$th component, and $N$ is the total number of molecules in unit volume. Also, Avogadro's number $\mathbf{N}$, is related to $N$ by

$$\mathbf{N}=N\frac{\Sigma x_iM_i}{d}, \qquad (3)$$

$M_i$ being the molecular weight of the $i$th component of the mixture whose density is $d$.

Thus, from (1), (2), and (3), we have

$$\frac{(2\varepsilon+1)(\varepsilon-n^2)}{3(2\varepsilon+n^2)}\cdot\frac{\Sigma x_iM_i}{d}=\frac{4\pi}{9kT}\mathbf{N}\Sigma x_i(\mu_{0i})^2 f_i g_i$$
$$=\Sigma x_i\,{}_0P_i f_i g_i, \qquad (4)$$

where ${}_0P_i$ is the orientation polarization of the $i$th component.

We shall now employ the same molecular model as that adopted in the abovementioned paper, that is, it will be assumed that the molecules can be represented by ellipsoids of internal dielectric constant $n_i^2$ and possessing permanent dipoles along one of the axes. If it be assumed that the surroundings of each ellipsoid are continuous and have a dielectric constant $\varepsilon$, then it can be shown (see Buckingham 1953) that

$$f_i=\frac{(2\varepsilon+1)[1+(n_i^2-1)A_i]}{3[\varepsilon+(n_i^2-\varepsilon)A_i]}, \qquad (5)$$

$$g_i=\frac{\varepsilon[1+(n_i^2-1)A_i]}{\varepsilon+(n_i^2-\varepsilon)A_i}, \qquad (6)$$

where $A_i$ is a shape factor and is determined by the relative lengths of the semi-axes. The $A_i$'s have been plotted as functions of the axial ratios by Osborn (1945).

From (4), (5), and (6) we find that

$$\frac{\varepsilon-n^2}{\varepsilon(2\varepsilon+n^2)}\,\frac{\Sigma x_i M_i}{d}=\Sigma x_i\, {}_oP_i\frac{[1+(n_i^2-1)A_i]^2}{[\varepsilon+(n_i^2-\varepsilon)A_i]^2}. \qquad (7)$$

In the special case of a two component system, the orientation polarization of the solute is given by

$${}_oP_2=\frac{1}{x_2}\left[\frac{\varepsilon+(n_2^2-\varepsilon)A_2}{1+(n_2^2-1)A_2}\right]^2\left\{\frac{\varepsilon-n^2}{\varepsilon(2\varepsilon+n^2)}\,\frac{\Sigma x_i M_i}{d}-x_1\,{}_oP_1\left[\frac{1+(n_1^2-1)A_1}{\varepsilon+(n_1^2-\varepsilon)A_1}\right]^2\right\}, \qquad (8)$$

where the subscripts 1 and 2 refer to the solvent and solute respectively.

There is still the question of the choice of the correct values for $n^2$, that is, the square of the effective refractive index of the solution, and for ${}_oP_1$. It will now be assumed that, for two component systems,

$$n^2=n_1^2+x_2(n_2^2-n_1^2)=n_2^2+x_1(n_1^2-n_2^2). \qquad (9)$$

While equation (9) will not necessarily exactly agree with experiment it is nevertheless a useful approximation, especially in the case of dilute solutions.

Smith (1952) has shown that the molecular polarization of a solvent is not independent of its concentration. We shall assume that the orientation polarization of the solvent varies linearly with concentration, so that

$${}_oP_1={}_oP_{\text{liquid}}+x_2\frac{\delta_oP_1}{\delta x_2}, \qquad (10)$$

that is, we have assumed that the rate of change of ${}_oP_1$ with $x_2$ is a co nstant derivatives higher than the first being neglected. This supposition is satisfactory when $x_2$ is small, that is, for dilute solutions, and it is in these that we are chiefly interested. $\delta_oP_1/\delta x_2$ may be evaluated in individual cases by means of data obtained from solutions of some standard substance dissolved in the polar solvent. The further assumption is now made that $\delta_oP_1/\delta x_2$ is independent of the nature of the solute, that is, the effective orientation polarization of the solvent will be considered to depend only upon its concentration. This somewhat crude assumption is probably the chief cause of any disagreement the theory may have with experiment. In equation (10), ${}_oP_{\text{liquid}}$ implies simply the orientation polarization of the pure liquid when computed by means of equation (7); $x_2$ will of course in this case be zero, so that

$${}_oP_{\text{liquid}}=\frac{(\varepsilon-n^2)[\varepsilon+(n^2-\varepsilon)A]^2M}{\varepsilon(2\varepsilon+n^2)[1+(n^2-1)A]^2d}. \qquad (11)$$

For the purpose of evaluating roughly the order of accuracy of the values for ${}_oP_2$, consider a mixture in which $A_1=A_2=1/3$, and $n^2=n_1^2=n_2^2$. Then

$${}_oP_2=\frac{1}{x_2}\left(\frac{2\varepsilon+n^2}{n^2+2}\right)^2\left[\frac{\varepsilon-n^2}{\varepsilon(2\varepsilon+n^2)}\,\frac{\Sigma x_i M_i}{d}-x_i\,{}_oP_1\left(\frac{n^2+2}{2\varepsilon+n^2}\right)^2\right],$$

that is,

$$x_2\,{}_oP_2=\left(\frac{2\varepsilon+n^2}{n^2+2}\right)^2\varphi,\ \text{say.}$$

If the solution being considered is similar to, for example, that of chlorobenzene in chloroform, with $\varepsilon=5$, $n^2=2\cdot5$, $x_2=0\cdot1$, ${}_oP_2=60$ c.c., and $\Sigma x_iM_i/d=100$ c.c., then $\varphi$ is of the order of $0\cdot8$ c.c., and for a 10 per cent. error in ${}_oP_2$, $\varphi$ must be known to $0\cdot08$ c.c.; since each term of $\varphi$ is approximately 4 c.c., an accuracy of 2 per cent. is required and ${}_oP_1$ must be known to $0\cdot7$ c.c. in 35 c.c., if the error be concentrated in this term.

When the solution has properties similar to those of chloroform in nitrobenzene, with $\varepsilon=30$, $n^2=2\cdot5$, $x_2=0\cdot1$, ${}_oP_2=20$ c.c., and $\Sigma x_iM_i/d=100$ c.c., then $\varphi$ is approximately $0\cdot01$ c.c. Assuming that all the error is in ${}_oP_1$, then for a 10 per cent. error in ${}_oP_2$, $\varphi$ must be known to $0\cdot001$ c.c., and since each term of $\varphi$ is of the order of $1\cdot5$ c.c., the required accuracy is $0\cdot07$ per cent., so that ${}_oP_1$ must be known to $0\cdot2$ c.c. in 330 c.c.

Thus we should expect the accuracy to be greatest when $\varepsilon$ is small and when ${}_oP_2$ is large. When only one of these conditions is satisfied, the order of accuracy might be expected to be fair, but if neither requirement is met, the errors could well be large.

## III. Application of the Method

Data suitable for testing the above theory have been tabulated by Jenkins (1934), Le Fèvre and Le Fèvre (1936*a*), and Le Fèvre and Russell (1936). The polar solvents employed were chloroform, chlorobenzene, and nitrobenzene, whilst the values of $\delta_oP_1/\delta x_2$ were found from the values for solutions of benzene in each of these solvents tabulated by Le Fèvre and Le Fèvre (1936*b*). In order that ${}_oP_1$ should be zero when benzene is the solute, the following equations giving the effective orientation polarization of the solvent were selected:

$$ {}_oP_{CHCl_3}=(17\cdot13-4\cdot49x_2)\ \text{c.c.,}$$
$$ {}_oP_{C_6H_5Cl}=(63\cdot26-2\cdot19x_2)\ \text{c.c.,}$$
$$ {}_oP_{C_6H_5NO_2}=(695\cdot0-247x_2)\ \text{c.c.}$$

In Table 1, the data and calculated moments are summarized. The results obtained when using non-polar solvents are included for comparative purposes. All results refer to solutions at 25 °C. The $n_i^2$ values employed were: $C_6H_6$, $2\cdot2725$ (when dealing with the values of Le Fèvre and Le Fèvre (1936*a*, 1936*b*), and $2\cdot2727$ for Jenkins's (1934) results; $CCl_4$, $2\cdot2277$; *n*-hexane, $1\cdot8870$; $CS_2$, $2\cdot6328$; $CHCl_3$, $2\cdot368$; $C_6H_5Cl$, $2\cdot554$; and $C_6H_5NO_2$, $2\cdot633$. The $A_i$'s used were: $CHCl_3$, $0\cdot46$; $C_6H_5Cl$, $0\cdot18$; and $C_6H_5NO_2$, $0\cdot17$ (for references see Buckingham 1953).

Values for the dielectric constants and densities of solutions of $(CH_2)_6N_4$, that is, hexamethylenetetramine, in chloroform at 25 °C have been tabulated by Le Fèvre and Rayner (1938). Since this molecule is believed to be spherically symmetrical one would expect it to show a zero moment. The solution possessing a mole fraction for the solute of $0\cdot019025$ (corresponding to a weight fraction of $0\cdot022267$) has a dielectric constant of $4\cdot6640$ and a density of $1\cdot47493$. By taking

$n^2$ for $(CH_2)_6N_4$ to be 2·75, one finds for the orientation polarization of this compound $-1\cdot6$ c.c. This result, in spite of its impossible sign, is by no means unsatisfactory, for small moments are invariably accompanied by large errors.

TABLE 1

DIPOLE MOMENTS CALCULATED FROM DATA FOR SOLUTIONS IN NON-POLAR AND POLAR SOLVENTS

| Solute | Solvent | $x_2$ | $\varepsilon$ | $d$ | $\mu_{02}$ (calc.)* |
|---|---|---|---|---|---|
| $C_6H_5NO_2$ | $C_6H_6$ | 0·016110 | 2·6390 | 0·88035 | 4·58 |
| | ,, | 0·020530 | 2·7411 | 0·88153 | 4·59 |
| | ,, | 0·023887 | 2·8067 | 0·88298 | 4·54 |
| | ,, | 0·01534 | 2·6224 | 0·8789 | 4·59 |
| | ,, | 0·02273 | 2·7894 | 0·8826 | 4·58 |
| | $CCl_4$ | 0·01845 | 2·6031 | 1·5775 | 4·52 |
| | ,, | 0·02444 | 2·7283 | 1·5751 | 4·53 |
| | *n*-Hexane | 0·01465 | 2·0896 | 0·6681 | 4·37 |
| | ,, | 0·04649 | 2·5508 | 0·6820 | 4·39 |
| | $CS_2$ | 0·00769 | 2·8857 | 1·2546 | 4·47 |
| | ,, | 0·01449 | 3·1105 | 1·2536 | 4·49 |
| | $CHCl_3$ | 0·039602 | 5·9669 | 1·45656 | 4·98 |
| | ,, | 0·062531 | 6·7001 | 1·44979 | 5·05 |
| | ,, | 0·090352 | 7·5569 | 1·44163 | 5·09 |
| | $C_6H_5Cl$ | 0·046199 | 6·5149 | 1·10560 | 4·87 |
| | ,, | 0·070873 | 7·0275 | 1·10808 | 4·89 |
| | ,, | 0·080073 | 7·2258 | 1·10904 | 4·96 |
| $C_6H_5Cl$ | $C_6H_6$ | 0·022973 | 2·3540 | 0·87968 | 1·64 |
| | ,, | 0·057267 | 2·4790 | 0·88848 | 1·76 |
| | ,, | 0·125128 | 2·7158 | 0·90559 | 1·76 |
| | $CHCl_3$ | 0·021182 | 4·7381 | 1·45836 | 1·79 |
| | ,, | 0·037554 | 4·7509 | 1·45125 | 1·81 |
| | ,, | 0·083665 | 4·7836 | 1·43090 | 1·78 |
| | $C_6H_5NO_2$ | 0·035649 | 33·490 | 1·19526 | 1·86 |
| | ,, | 0·061809 | 32·539 | 1·19277 | 2·15 |
| | ,, | 0·106235 | 30·796 | 1·18865 | 1·91 |
| $CHCl_3$ | $C_6H_6$ | 0·065875 | 2·3965 | 0·90894 | 0·99 |
| | ,, | 0·089280 | 2·4406 | 0·92175 | 0·98 |
| | ,, | 0·147558 | 2·5541 | 0·95336 | 0·98 |
| | $C_6H_5Cl$ | 0·062172 | 5·5804 | 1·11952 | 0·96 |
| | ,, | 0·096741 | 5·5588 | 1·13006 | 0·94 |
| | ,, | 0·123919 | 5·5482 | 1·13850 | 0·96 |
| | $C_6H_5NO_2$ | 0·092818 | 32·250 | 1·22028 | 1·53 |
| | ,, | 0·148910 | 30·673 | 1·23386 | 1·48 |
| | ,, | 0·264948 | 27·372 | 1·26256 | 1·46 |

* The observed $\mu_{gas}$ values are: $C_6H_5NO_2$, 4·24; $C_6H_5Cl$, 1·73; and $CHCl_3$, 1·01. References for the above values are as follows: the first three solutions of $C_6H_5NO_2$ in $C_6H_6$ are from Le Fèvre and Le Fèvre (1936*a*), while the remaining data for $C_6H_5NO_2$ in non-polar solvents are from Jenkins (1934). The other results are those of Le Fèvre and Russell (1936).

## IV. Application to Aqueous Solutions

As there has been a need for a method whereby dipole moments can be computed from data obtained from aqueous solutions, it seemed of interest to test the applicability of equation (**8**) to systems of this type. Because $\varepsilon$ is large, the accuracy might be expected to be poor, but if the moment of the solute is large (as is the case for zwitterions such as glycine in water), then the results should be reasonably satisfactory.

The following constants were used for water at 25 °C: $n^2=1\cdot861$, $A=0\cdot34$, and ${}_0P_1=182\cdot6+85\cdot7x_2$ (for references see Angyal and Le Fèvre 1952). The equation for ${}_0P_1$ was derived by taking the dielectric constant of water to be 78·48 at 25 °C (see Albright 1937), so that the orientation polarization of water is calculated to be 182·6 c.c. by equation (**11**), using a density of 0·99707 g/c.c. Albright's (1937) data, giving the densities and dielectric constants of mixtures of acetone and water of various concentrations, enable us to calculate that the effective orientation polarization of the water in the most dilute solution shown, is $185\cdot4_6$ c.c. This result was obtained by assuming that the orientation polarization of acetone is $51057/T$ c.c., and that $n_2^2$ is 1·995. Since the solution mentioned above had an acetone mole fraction of 0·033354, one finds the above result for ${}_0P_1$ if it be assumed that the relationship between ${}_0P_1$ and $x_2$ is linear when $x_2$ is small.

The dielectric increment of pyridine in water, that is, the rate of change of $\varepsilon$ with the concentration of pyridine (expressed in mol/l.) at zero concentration, is $-4\cdot2$ at 25 °C, according to Devoto (1933). The densities of several solutions of pyridine in water at 25 °C have been tabulated by Hartley, Thomas, and Applebey (1908). Table 2 has been compiled as a result of calculations employing the values of these authors.

Table 2

Dipole moments for pyridine calculated from solutions in water at 25 °C

| $x_2$ | $\varepsilon$ | $d$ | $\mu_0$ (calc. by (**8**)) | Probable $\mu_{gas}$ |
|---|---|---|---|---|
| 0·02444 | 73·22 | 0·99953 | 3·1 | 2·5 |
| 0·05159 | 68·23 | 1·00117 | 3·1 | 2·5 |

The probable $\mu_{gas}$ value was determined from the value of Middleton and Partington (1938) for the dipole moment of pyridine in benzene solution, namely, 2·26D, by the application of equation (**38**) of Buckingham (1953). The $n^2$ value used for pyridine was 2·272, while $A$ was taken to be 0·24.

Although the results of Table 2 are far from ideal, they are nevertheless not disappointing, for there are uncertainties underlying this example. Thus

the values of Devoto, which are almost certainly high for glycine in water (see Lindquist and Schmidt 1938) may not be precise. Again, Hartley, Thomas, and Applebey (1908) suggest, on the basis of an observed contraction in volume and evolution of heat on mixing pyridine and water, that a hydrate may be formed. The moment of the hydrate, if it exists, would not, of course, be the same as that of pure pyridine.

There are in the literature the results of many determinations of the dielectric constants of solutions of glycine in water. The values of Hedestrand (1928), Wyman and McMeekin (1933), and Lindquist and Schmidt (1938) are in approximate agreement and the last mentioned authors' values have been used below. The density of any solution of glycine in water at 25 °C can be evaluated from Albright's (1937) formula, namely,

$$d=0\cdot 9971+0\cdot 0320c-0\cdot 0010c^2,$$

where $c$ is the concentration of the amino acid in mol/l. Thus the $c$'s of Lindquist and Schmidt may be converted into mole fractions. $n^2$ for glycine was estimated from Vogel's (1948) list of group refractivities, and a molecular volume of 43·3 c.c. obtained from the values of Dalton and Schmidt (1933). The shape of the glycine molecule in a crystal has been illustrated by Wyckoff (1951), and with obvious alterations required by the dipolar-ionic structure, an estimate of the molecular " dimensions " can be made. If we take the dipole axis to be the line joining the N-atom to the point mid-way between the two O-atoms, then we find that $A=6\cdot 08$, $B=4\cdot 64$, and $C=3\cdot 82$ (see Barclay and Le Fèvre 1950), whence the shape factor $A=0\cdot 24$. The actual charge separation in the dipolar-ionic form is 2·95 Å.

Thus, for glycine in water at 25 °C, we have $x_2=0\cdot 018553$ (corresponding to a concentration of 1 mol/l.), $\varepsilon=101\cdot 41$, and $d=1\cdot 0281$ g/c.c. From the above values one finds that ${}_0P_2=3607$ c.c., whence $\mu_0=13\cdot 3$D. This result is in good agreement with the value calculated by multiplying the electronic charge, that is, $4\cdot 802\times 10^{-10}$ e.s.u., by the charge separation (estimated to be $2\cdot 95\times 10^{-8}$ cm), namely, 14·2 D. From theoretical considerations and from a curve showing the solubility of glycine in alcohol-water mixtures at 25 °C, Kirkwood (1934) calculated a moment of 15·0 D for the glycine molecule, whose shape he took to be spherical.

In order to examine further the validity of equation (8), it has been applied to data obtained from solutions of β-alanine (that is, β-aminopropionic acid) in water at 25 °C. The results of Wyman and McMeekin (1933) indicate that the dielectric constant of a molar solution is 113·04. The density measurements of Gucker and Allen (1942) enable us to calculate that a molar solution has a β-alanine mole fraction of 0·018849 and a density of 1·0269 g/c.c. These authors' value for the molecular volume, namely $58\cdot 72_3$ c.c. and a molecular refraction computed from Vogel's list lead to an $n^2$ value of 2·66.

For the purpose of evaluating the molecular dimensions, the following model (Fig. 1), showing interatomic distances (Å) and angles was employed :

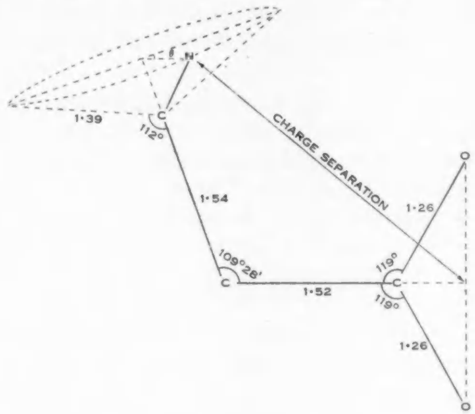


Fig. 1

The actual charge separation is therefore

$$\{[(1\cdot52+1\cdot26\cos 61°)\sin 70°\,32']^2+[1\cdot39\sin 68°]^2-2[(1\cdot52+1\cdot26\cos 61°)\sin 70°\,32'][1\cdot39\sin 68°]\cos\theta+[(1\cdot52+1\cdot26\cos 61°)\cos 70°\,32'+1\cdot54+1\cdot39\cos 68°]^2\}^{\frac{1}{2}},$$

which is equal to $\sqrt{(13\cdot38-5\cdot17_7\cos\theta)}$. Assuming that the $(NH_3)^+$ group is free to rotate about the C—C bond, the mean value for the charge separation is

$$\frac{1}{\pi}\int_0^\pi\sqrt{(13\cdot38-5\cdot17_7\cos\theta)}d\theta,$$

which, on putting $\theta$ equal to $(\pi-2\varphi)$, becomes

$$\frac{8\cdot616}{\pi}\int_0^\pi\sqrt{(1-0\cdot558\sin^2\varphi)}d\varphi.$$

This expression is a " complete elliptic integral of the second kind ", and from tables the mean charge separation may be calculated to be 3·62 Å. The shape factor $A$ is then found to be 0·22.

Thus, using equation (8), one finds for β-alanine an orientation polarization of 6270 c.c., whence a dipole moment of 17·5 D. The moment calculated by multiplying the electronic charge by $3\cdot62\times10^{-8}$ is 17·4 D.

## V. Discussion

The results of Table 1 show that the method is most suitable in those cases when the dielectric constant of the polar solvent is small, and is not accurate when the dipole moment of the solute is small. The application of the theory to

solutions of glycine and β-alanine in water shows that moments which seem most satisfactory can be predicted for this class of compound. The necessary requirement that the moment of the solute be appreciable is not peculiar to this new approach, it being well known that small moments are difficult to determine accurately; Everard, Kumar, and Sutton (1951) state that "moments less than 0·4 D are indistinguishable from zero".

In spite of its limitations therefore, the above theory does provide a means whereby "true" dipole moments may be computed from data obtained from solutions in polar solvents. The success of its application to solutions of highly polar compounds in water is most encouraging, for in the past no reliable method of calculating the moments of proteins and similar compounds soluble only in solvents of high dielectric constant, has been available.

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# A POLAROGRAPHIC INVESTIGATION OF THE BINDING PROPERTIES OF SERUM ALBUMIN

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*Summary*

It has been shown that the binding capacity of dissolved serum albumin for methyl orange increases on dilution. The increased binding capacity implies a change in the protein molecule. The possible nature of this change is discussed.

## I. Introduction

Although various aspects of the interaction of dyes and proteins have been extensively investigated during the last 25 years, there have been few references to an increased binding capacity of the protein molecule in dilute solution. Klotz and Urquhart (1949) noted the greater binding power of a 0·2 per cent. albumin solution compared with a 1 per cent. solution, and attributed the difference to a change in the activity coefficients of the various albumin-dye complexes with changing total albumin concentration. Carroll (1950), studying the action of enzymes on dye-coated albumin molecules, made a similar observation but did not discuss the effect. The same is true of Laurence (1952), who used the method of polarized fluorescence (Weber 1952).

In these Laboratories, the phenomenon was observed in the course of an investigation of the binding of methyl red by normal horse serum (Breyer and Radcliff, unpublished data), and was thought to indicate a change in surface area of the albumin component of the serum.

The purpose of the present investigation was to study this phenomenon in detail, with a view to elucidating the changes in the albumin molecule which bring about these differences in the binding capacity.

The extent of binding was measured polarographically, following the method proposed by Breyer (1938).

## II. Methods and Materials

The polarograph used was of the conventional manual type.

Considerable difficulties were experienced at first because the capillaries developed irregularities of drop formation when immersed in protein solutions. It was found, however, that the difficulties could be completely avoided by treating the capillaries with an ethereal solution of silicone and subsequent hydrolysis by immersion in water. This procedure is best repeated twice in succession. It is important that the capillary should be kept dropping during treatment, in order to avoid blocking the orifice. It might be necessary to repeat the application of silicone after several months.

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The buffer solution contained acetate, phosphate, and borate (Prideaux and Ward 1924). All experiments were carried out at 25 °C. Complete removal of air from albumin solutions required upwards of half an hour, since nitrogen could be bubbled through only at a slow rate to avoid excessive foaming.

The addition of crystalline serum albumin in the concentrations used did not alter the viscosity of the solutions appreciably, and no correction of the diffusion current was found necessary. Methyl orange was recrystallized from ethanol. Crystalline bovine plasma albumin, fraction V, was obtained from the Armour Laboratories, Chicago, U.S.A.

## III. Results

### (a) *The Effect of Albumin on the Diffusion Current of Methyl Orange*

The polarographic diffusion current of the dye in the presence of albumin ($(i_d)_P$) was lower than that in the absence of albumin ($i_d$) for the same dye concentration. Further, with increasing albumin concentration, the ratio $(i_d)_P/i_d$ became progressively lower, tending asymptotically to a limiting value ($q$) (see Tables 1 and 2).

Table 1

BINDING OF METHYL ORANGE BY SERUM ALBUMIN

pH 4·9; ionic strength 0·07

| Albumin Concentration (% w/v) | $(i_d)_P/i_d$ | | | | Number of Binding Sites $c_B/c_fc_P$ (arbitrary units) |
|---|---|---|---|---|---|
| | Total Dye Concentration | | | Mean | |
| | $2\cdot4\times10^{-5}$M | $6\cdot1\times10^{-5}$M | $2\cdot4\times10^{-4}$M | | |
| 0·005 | 0·934 | 0·923 | 0·890 | 0·916 | 3·0 |
| 0·007 | 0·854 | 0·877 | 0·834 | 0·855 | 4·1 |
| 0·01 | 0·821 | 0·866 | 0·775 | 0·821 | 3·8 |
| 0·02 | 0·700 | 0·703 | 0·685 | 0·696 | 4·5 |
| 0·03 | 0·746 | 0·705 | 0·696 | 0·716 | 2·6 |
| 0·05 | 0·751 | 0·674 | 0·681 | 0·702 | 1·7 |
| 0·1 | 0·680 | 0·674 | 0·648 | 0·667 | 1·04 |
| 0·2 | 0·577 | | 0·545 | 0·561 | 1·07 |
| 0·4 | 0·511 | | 0·472 | 0·492 | 0·95 |
| 0·6 | 0·465 | | 0·425 | 0·445 | 1·03 |
| 0·8 | 0·427 | | 0·395 | 0·411 | |
| 1·2 | 0·394 | | 0·381 | 0·388 | |
| 1·6 | 0·380 | 0·373 | 0·363 | 0·372 | |

Extrapolated, $q=0\cdot35$

The lowering of the diffusion current of the dyestuff was due to the protein-dye interaction, not, as assumed by Keilin (1948), to adsorption of protein onto the electrode. This has been shown previously by Breyer (1938) in experiments where a more strongly bound substance displaced the dye from the protein surface, so that the diffusion current of the dye in presence of protein ($(i_d)_P$)

rose to its original value ($i_d$). Pfankuch and Hagenguth (1942), in a study of the binding of heavy metal ions by proteins, also showed that lowering of the diffusion current of the ions was due to complex formation with the protein, and not to viscosity changes or adsorption of protein onto the mercury drop. Finally, a series of experiments using the equilibrium dialysis method (Klotz, Walker, and Pivan 1946), carried out during the present investigation, confirmed that the lowering of the diffusion current in the presence of protein was due to the protein-dye interaction (see Section III (*d*)).

Table 2

BINDING OF METHYL ORANGE BY SERUM ALBUMIN

pH 7·3, ionic strength 0·14

| Albumin Concentration (% w/v) | $(i_d)_P/i_d$ | | | | | | Number of Binding Sites $c_B/c_f c_P$ (arbitrary units) |
|---|---|---|---|---|---|---|---|
| | Total Dye Concentration | | | | | Mean | |
| | $2\cdot4\times10^{-5}$M | $6\cdot1\times10^{-5}$M | $1\cdot2\times10^{-4}$M | $1\cdot7\times10^{-4}$M | $2\cdot4\times10^{-4}$M | | |
| 0·003 | 0·918 | 0·920 | 0·895 | | | 0·911 | 4·85 |
| 0·005 | 0·834 | 0·837 | 0·896 | 0·853 | 0·883 | 0·861 | 4·95 |
| 0·007 | 0·806 | 0·930 | 0·858 | | | 0·865 | 3·4 |
| 0·01 | 0·828 | | 0·822 | 0·848 | 0·829 | 0·832 | 3·2 |
| 0·02 | 0·779 | 0·745 | 0·794 | | | 0·773 | 2·4 |
| 0·03 | 0·778 | | 0·743 | 0·759 | 0·770 | 0·763 | 1·7 |
| 0·05 | 0·718 | 0·734 | 0·728 | 0·737 | 0·750 | 0·733 | 1·23 |
| 0·1 | 0·685 | 0·678 | 0·672 | 0·680 | 0·674 | 0·678 | 0·85 |
| 0·2 | 0·591 | 0·628 | 0·595 | 0·562 | 0·595 | 0·595 | 0·69 |
| 0·4 | 0·508 | 0·539 | 0·529 | 0·485 | 0·465 | 0·505 | 0·61 |
| 0·6 | 0·442 | 0·443 | 0·448 | 0·414 | 0·410 | 0·431 | 0·72 |
| 0·8 | | | 0·400 | 0·400 | 0·377 | 0·392 | 0·83 |
| 0·9 | 0·381 | 0·389 | 0·392 | | | 0·387 | 0·78 |
| 1·2 | 0·354 | 0·366 | 0·348 | 0·342 | 0·343 | 0·351 | |
| 1·6 | 0·309 | 0·332 | 0·306 | 0·325 | 0·314 | 0·317 | |

Extrapolated, $q=0\cdot30$

### (*b*) *Calculation of the Extent of Interaction*

The limiting value of $(i_d)_P$ at excess albumin concentrations represents the polarographic current due to reduction of dye-stuff, when all the latter is bound to the albumin. At concentrations of albumin where only part of the dye is bound, the diffusion current observed can be expressed as (after Tanford 1951)

$$(i_d)_P=A(c_f+qc_B), \qquad (1)$$

where $c_f$=concentration of free dye,

$c_B$=concentration of bound dye,

$q$=limiting value of $(i_d)_P/i_d$,

and $A$=the Ilkovič constant (Ilkovič 1934).

In the absence of albumin and for the same total dye concentration ($c_T$),

$$i_d = Ac_T, \qquad \text{(2)}$$

where

$$c_T = c_f + c_B$$

of equation (1).

It follows from equations (1) and (2) that

$$\frac{c_B}{c_f} = \frac{1-(i_d)_P/i_d}{(i_d)_P/i_d - q}. \qquad \text{(3)}$$

*(c) Calculation of the Total Number of Binding Sites* (n) *on the Albumin Molecule*

The protein-dye interaction can be represented by the equilibrium

$$A + B \rightleftharpoons C, \qquad \text{(4)}$$

where $A$ denotes free dyestuff, $B$ free binding sites, and $C$ occupied binding sites.

The equilibrium constant ($K$) of the reaction is given by

$$K = \frac{[C]}{[A][B]}, \qquad \text{(5)}$$

or

$$K = \frac{c_B}{c_f(nc_p - c_B)}, \qquad \text{(6)}$$

where $c_P$ is the total concentration of albumin.

From equation (6) the total number of binding sites results as

$$n = \frac{c_B(1/K + c_f)}{c_f c_P}.* \qquad \text{(7)}$$

It has been found (Tables 1 and 2) that, for a given concentration of albumin, $(i_d)_P/i_d$ is constant within experimental error for all concentrations of methyl orange in the range investigated. It follows then from equation (3) that, at a given protein concentration, $c_B/c_f$ and hence $c_B/c_f c_P$ are also constant. Since $n$, the total number of binding sites on the albumin molecule, will not vary with the concentration of dyestuff, it follows from equation (7) that $1/K + c_f$ must also be independent of the dye concentration ; consequently $C_f$ must be negligibly small compared with $1/K$, and equation (7) may be written as

$$n = \frac{c_B}{Kc_f c_P}. \qquad \text{(8)}$$

It is reasonable to assume that $K$ is constant for all values of $c_B$, $c_f$, and $c_P$, in other words, that all binding sites on the albumin molecule are equivalent (Klotz and Urquhart 1949), although Scatchard, Sheinberg, and Armstrong (1950), and Karush and Sonenberg (1949) have pointed out that this assumption can

* A more rigorous treatment of the kinetics of the interaction, involving consideration of the various dye-albumin complexes, leads to the same expression for $n$ (cf. Klotz, Walker, and Pivan 1946).

only be regarded as an approximation. Since the value of $K$ is not known, it is not possible to calculate the absolute values of $n$. However, the term $c_B/c_f c_P$ is a linear function of $n$, and it is possible to calculate the *relative* numbers of available binding sites per albumin molecule at different albumin concentrations.

### (*d*) *Equilibrium Dialysis*

In the equilibrium dialysis experiments, the albumin solution was contained in a cellophane sac which was immersed in the appropriate buffer solution. The requisite amount of dyestuff was added, in some cases to the outside buffer solution, in others to the protein solution inside the cellophane sac. The system was kept at 25 °C until equilibrium had been attained (usually about 3 days). Polarograms were then taken of the solutions inside and outside the membrane.

TABLE 3

BINDING OF METHYL ORANGE BY SERUM ALBUMIN

pH 7·3, ionic strength 0·14 ; calculation of $q$ from dialysis experiments

| Albumin Concentration (% w/v) | $\frac{(i_d)_P}{i_d}$ | $(i_d)_1$ | $(i_d)_2$ | $q$ |
|---|---|---|---|---|
| 0·2 | 0·595 | 0·335 | 0·431 | 0·246 |
| | | 0·167 | 0·247 | 0·323 |
| 0·6 | 0·431 | 0·283 | 0·571 | 0·275 |
| | | | Mean .. | 0·28 |

The cellophane membrane is permeable to dyestuff but not to protein. At equilibrium, the concentration of dye outside the membrane is equal to the concentration of unbound dye ($c_f$) inside the membrane.

Let $(i_d)_1$ be the polarographic current of the solution inside the sac and $(i_d)_2$ be the polarographic current of the solution outside the sac. Then, in the previously defined notation,

$$(i_d)_1=(i_d)_P=A(c_f+qc_B), \qquad (9)$$

and

$$(i_d)_2=Ac_f. \qquad (10)$$

Since

$$i_d=Ac_T, \qquad (2)$$

$q$ is given by

$$q=\frac{(i_d)_1-(i_d)_2}{(i_d)_1/[(i_d)_P/i_d]-(i_d)_2}. \qquad (11)$$

The values of $q$ obtained (Table 3) were in good agreement with the value of $q$ derived by extrapolation of the ratio $(i_d)_P/i_d$ (Table 2), an independent proof of the validity of the calculation of $c_B/c_f$ by means of equation (3).

## IV. Discussion

The values of $c_B/c_f c_P$ in Tables 1 and 2 show that the number of binding sites on the albumin molecule increases considerably at low concentrations of albumin. This increase might be due either to a dissociation of the molecule into smaller fragments, or to some other process, as for instance, unfurling of the protein chains, unmasking of binding sites, etc. as discussed in the following sections.

### (a) *Dissociation*

The dissociation of the protein molecule into smaller subunits has been postulated (*inter alia*) by Lundgren (1936), Pedersen (1936), Johnson (1946), and Gutfreund (1952). To find out whether in the present case dissociation is responsible for the increased binding capacity of the protein, it seemed best to investigate theoretically the increase in surface area of the albumin molecule on an assumed splitting into smaller units. The simplest and most plausible models for a possible dissociation of the albumin molecule are discussed below.

(1) Consider the albumin molecule to be a sphere, which dissociates into $x$ equal, smaller spheres. Let $r_u$ be the radius of the undissociated molecule, and $r_D$ be the radius of the subunits formed. Then, if dissociation is complete, and the density of the albumin is unchanged,

$$\frac{4}{3}\pi r_u^3 = x\frac{4}{3}\pi r_D^3,$$

and

$$r_u = x^{1/3} r_D.$$

$$\text{Surface area before dissociation} = 4\pi r_u^2,$$

$$\text{surface area after dissociation} = x4\pi r_D^2,$$

and

$$\text{increase in surface area on dissociation} = x^{1/3}.$$

(2) Consider the albumin molecule as a cylinder, radius of the base $r$, and height $8r$ (cf. Neurath and Saum 1939; Neurath 1940; Neurath, Cooper, and Erickson 1942). Dissociation is supposed to occur by the splitting of this molecule into $x$ particles:

(i) Parallel to the base of the cylinder

$$\text{Surface area before dissociation} = 18\pi r^2,$$

$$\text{surface area after dissociation} = 18\pi r^2 + (x-1)2\pi r^2,$$

and

$$\text{increase in surface area on dissociation} = (8+x)/9.$$

(ii) Perpendicular to the base of the cylinder

$$\text{Surface area before dissociation} = 18\pi r^2,$$

$$\text{surface area after dissociation} = 18\pi r^2 + 2xr \times 8r,$$

and

$$\text{increase in surface area on dissociation} = (9\pi + 8x)/9\pi.$$

If the albumin molecule does dissociate into a number of separate fragments ($x$), then the rate of increase of surface area with $x$ should be somewhere between the values calculated for models (1) and (2 (ii)) (Table 4). Now, at pH 4·9 (Table 1), the binding capacity of the albumin molecule at a concentration of 0·02 per cent. is 3·8 times that at a concentration of 0·1 per cent.; at pH 7·3 (Table 2), the binding capacity at a concentration of 0·005 per cent. is 6·8 times that at a concentration of 0·2 per cent. Thus, if dissociation were responsible, the magnitude of the increase in binding capacity at pH 4·9 would imply a splitting of the molecule into at least 10, and at pH 7·3 into at least 20 separate fragments. It is most improbable that such a large number of separate particles could be formed on mere dilution.

TABLE 4

SURFACE INCREASE ON DISSOCIATION

| Number of Particles $x$ | Increase in Surface Area according to Model | | |
|---|---|---|---|
| | (1) | (2 (i)) | (2 (ii)) |
| 2 | 1·26 | 1·11 | 1·56 |
| 3 | 1·44 | 1·22 | 1·84 |
| 4 | 1·59 | 1·33 | 2·12 |
| 5 | 1·71 | 1·44 | 2·40 |
| 6 | 1·82 | 1·56 | 2·68 |
| 8 | 2·00 | 1·78 | 3·23 |
| 10 | 2·15 | 2·00 | 3·80 |
| 20 | 2·71 | 3·33 | 6·65 |

### (*b*) *Processes other than Dissociation*

The exact nature of the binding groups on the albumin molecule is not known as yet, and it is not possible, therefore, to attempt a detailed interpretation of the observed increase in binding capacity. It can be said only that a change in the molecule occurs, and that this is not simply a dissociation process. Some of the possible changes are briefly mentioned below.

On dilution, unfurling, or unfolding, of the albumin molecule may take place (as in the case of urea-denaturation, Neurath and Saum (1939)), whereby a greater number of binding sites become exposed. Or the change may be a swelling, or expansion, of the molecule, resulting in a larger area becoming available to dye molecules, and perhaps even allowing the latter to penetrate into the structure of the molecule. Such a process has been postulated for the human albumin molecule (Klotz, Burkhard, and Urquhart 1952) under conditions where the negative charge on the molecule exceeds 10.

These processes would make available binding sites which previously were sterically hindered or masked. On the other hand, it is possible that the change

is not so much a structural one, but rather involves the breaking of bonds (most probably hydrogen bonds), at the surface of the protein, making more groups on the molecule available as active centres for the binding of dyestuff.

### (c) *Comparison of Results at Different pH*

The number of available binding sites on the albumin molecule ($n$) is given by

$$n=\frac{c_B}{Kc_fc_P}. \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (8)$$

Thus $c_B/c_fc_P$ is a linear measure of $n$ under conditions where $K$, the equilibrium constant of the interaction, does not change. This is the case in a series of experiments at fixed pH, where only the total concentrations of dye and albumin change.

If, however, the pH is changed, then $K$ will change also. Therefore, the expression $c_B/c_fc_P$ cannot be used to compare the number of binding sites in the molecule in solutions of different pH.

There are, however, some points of interest apparent from a comparison of the results obtained at different pH. At pH 4·9 the binding capacity of the albumin molecule reaches a limiting value already at a concentration of 0·02 per cent., whereas at pH 7·3 the limiting value is reached only at 0·005 per cent. Finally, it seems noteworthy that at pH 7·3 the binding capacity increases on dilution to a greater extent (6·8 times) than at pH 4·9 (3·8 times).

## V. Conclusion

A fuller theoretical evaluation of the experimental results reported in this paper must await further development of protein chemistry. In the meantime, however, polarographic investigation of adsorption processes promises to be of value in the study of protein structure, since the measurement of available binding sites affords information not directly forthcoming from the standard physical methods of ultra-centrifugation, electrophoresis, and osmotic pressure measurements. Moreover, the latter techniques are not satisfactory for protein concentrations as low as 0·005 per cent.

## VI. Acknowledgment

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# INFRA-RED SPECTRA OF COMPOUNDS CONTAINING THE AZO-GROUP

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*Summary*

Infra-red absorption spectra, over the rock-salt region, are reported for 43 substances believed to contain the —N=N— group. In the " double bond " region two common frequencies are noted, at 1406 and 1579 $cm^{-1}$ (with standard deviations of $\pm 14$ and $\pm 9$ $cm^{-1}$ respectively). Since these may arise wholly or partly from vibrations of the aromatic or heterocyclic nuclei adjacent to the azo-linkages, the conclusion is drawn that N=N stretching modes, if active in absorption, are contributors to either or both of the bands at the points mentioned.

## I. Introduction

The infra-red or Raman spectra of molecules containing the azo-group have been, until now, little studied. Herzberg's (1945) monograph quotes one example only—that of azomethane—provided by the observations of West and Killingsworth (1938); it omits comparable (Raman) data for solutions in carbon tetrachloride which appeared during the same year from Kahovec *et al.* (1938). Barnes, Liddel, and Williams (1943) published, without discussion, a small spectrogram of azobenzene taken between 1200 and 1700 $cm^{-1}$ thus amplifying a rather primitive report by Coblentz (1905); more recently Tetlow (1950) has recorded the absorption bands from 600 to 1400 $cm^{-1}$ of both the *cis*- and *trans*-isomers of this substance, while its Raman spectrum has been described by Imanishi and Kanda (1949) and by Stammreich (1950). During a wide investigation of hydrogen-bond formation, Hendricks *et al.* (1936) examined incidentally a few *o*-hydroxyazo- dyes in the region around 7000 $cm^{-1}$. In their book Randall *et al.* (1949) list —N=N— against $6 \cdot 15$–$6 \cdot 35 \mu$ without further details. Together with the infra-red spectra of certain aromatic diazocyanides, reproduced in the papers of Anderson, Le Fèvre, and Savage (1947) and Sheppard and Sutherland (1947), the above constitutes the literature on the subject. Its slightness is evident, and has been the reason for making a survey the results of which, covering the infra-red absorption of some 40 assorted azo-compounds, form the subject of this paper.

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## II. Experimental

The substances used have been prepared by the methods described or cited in the following references:

| | |
|---|---|
| Diazocyanides | Anderson, Le Fèvre, and Savage (1947) |
| Diazosulphonates | Freeman and Le Fèvre (1951) |
| Diazosulphones | Freeman *et al.* (1952) |
| Azoxysulphones | Freeman *et al.* (1952) |
| Diazotates | Beilstein's Handbuch, Vol. 16 |
| Diazocarboxyamides | Freeman, Le Fèvre, and Wilson (1951) |
| Triazens | Le Fèvre and Liddicoet (1951) |
| Diazoaminobenzene | Le Fèvre and Vine (1937) |
| Azobenzenes | Hartley and Le Fèvre (1939) |
| Azopyridine | Le Fèvre and Worth (1951) |

In most cases specimens have been examined between 600 and 1800 $cm^{-1}$ as mulls in " Nujol " with a Perkin-Elmer spectrometer, model 12C, having rock-salt optics and a Brown recorder, frequency calibration being effected by the ammonia and atmospheric absorption features listed by Oetjen, Kao, and Randall (1942) for an NaCl prism. After subtraction of the " Nujol " " background " the curves shown as Figure 1 were obtained. In two instances, the sodium and potassium *o*-chlorobenzenediazosulphonates, a marked broadening of the paraffinic absorption around 1455 $cm^{-1}$ was noted. This occurrence has been indicated by dotted lines in the spectra of these two salts. All compounds whose names are not preceded by *cis*- or *trans*- are *trans*- forms.

## III. Assignments of Main Bands

The chief absorptions, as read from the original curves, are listed in Table 1; since they have been obtained from layers pressed between rock-salt " flats ", their relative intensities can be judged only within any one spectrum of Figure 1. The assignments suggested in the right-hand columns are discussed later.

## IV. Discussion of Results

### (*a*) *General*

Nearly all the compounds show a strong band around 700 $cm^{-1}$ with a strength reminiscent of the $\nu_4{}^{\perp H}$ fundamental vibration of benzene (671 $cm^{-1}$ in the hydrocarbon itself: Herzfeld, Ingold, and Poole 1946). Additional absorptions are often in evidence from 700–740 $cm^{-1}$ with mono-substituted, and in the region from 800–840 $cm^{-1}$ with *p*-disubstituted benzene derivatives (Thompson and Whiffen 1945; Barnes *et al.* 1948). Most of the substances absorb also through the range 770–790 $cm^{-1}$; such features might be related to the difference tone (1178 − 405 = 773 $cm^{-1}$) observed by Herzfeld, Ingold, and Poole, although where they occur with either chloro- or *o*-derivatives they cannot be distinguished with certainty from those at 720–790 $cm^{-1}$ or 760–770 $cm^{-1}$ respectively to be expected for such structures. Nevertheless, it seems worth pointing out that among the three pairs of position isomerides, namely, the chlorobenzenediazocyanides, and the chlorobenzene- and iodobenzenediazosulphonates, included in Table 1 there is always an absorption

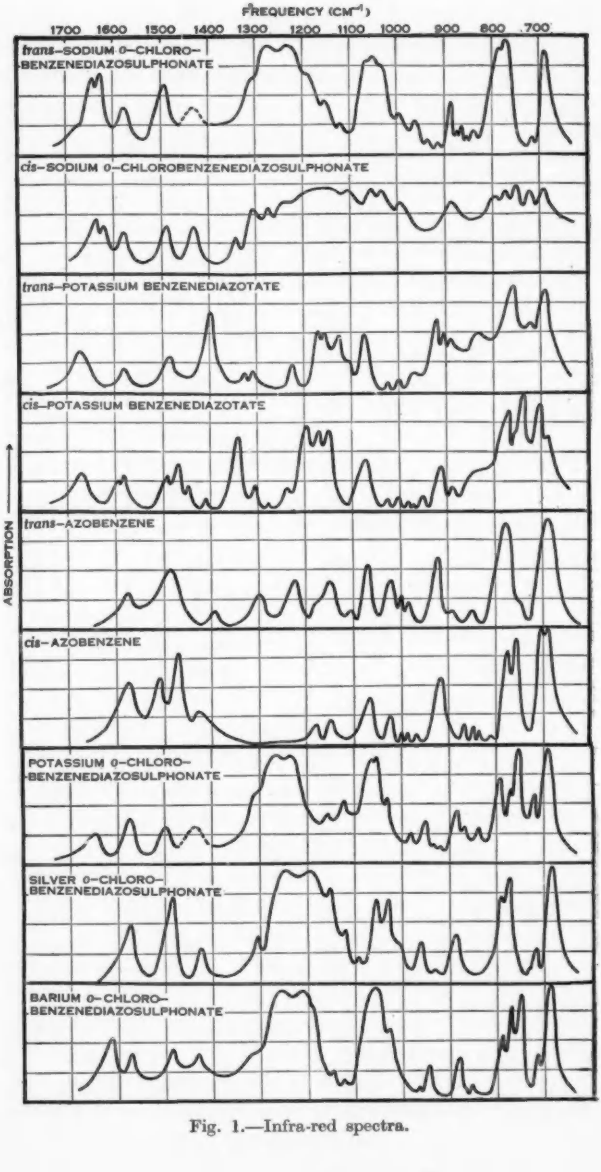


Fig. 1.—Infra-red spectra.

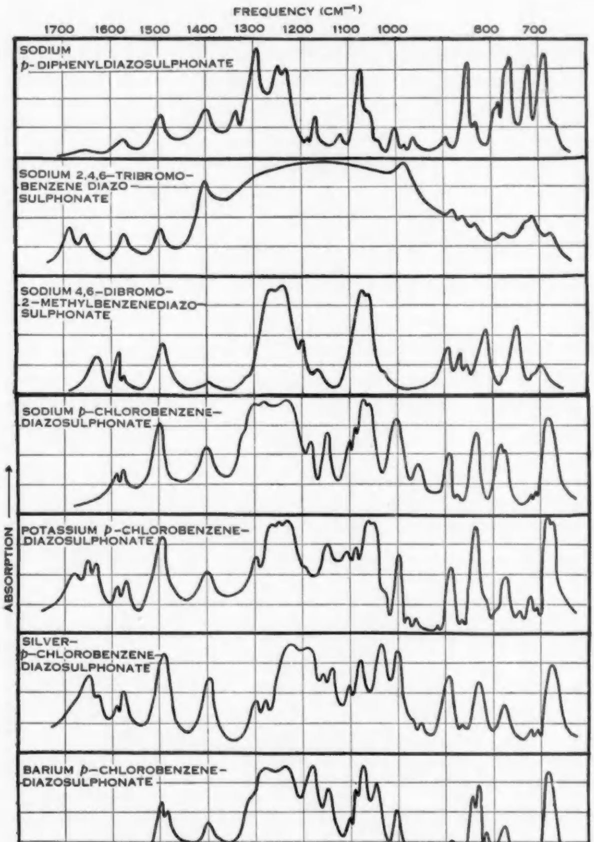


Fig. 1 (*Continued*)

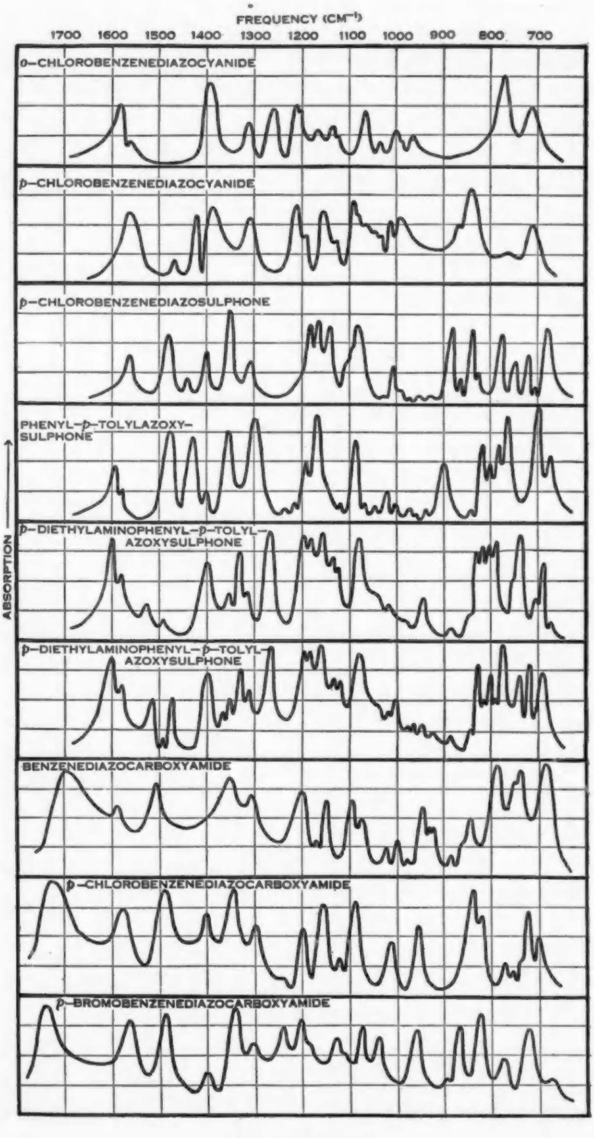


Fig. 1 (*Continued*)

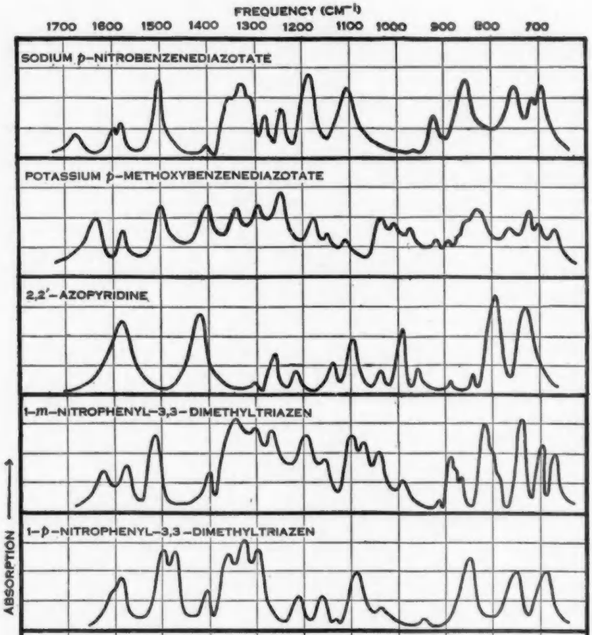


Fig. 1 (*Continued*)

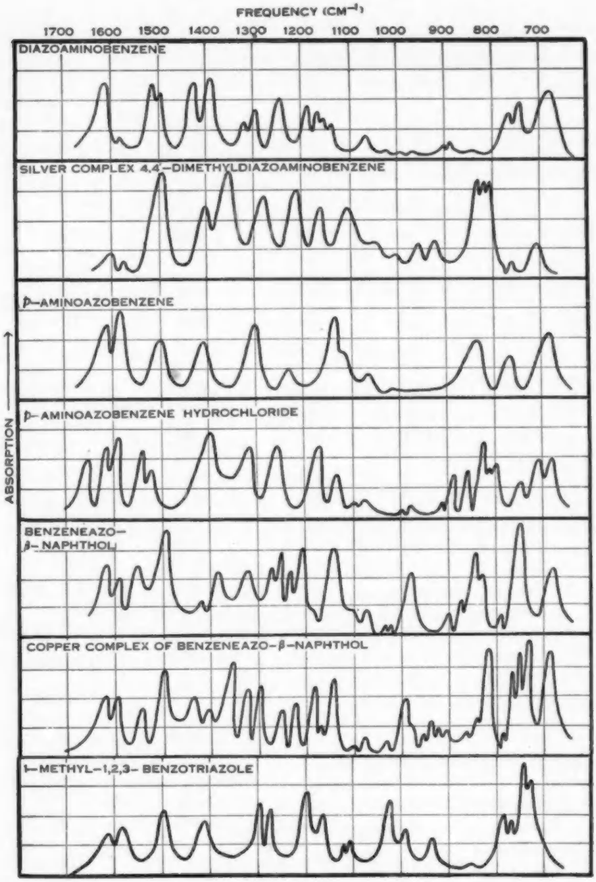


Fig. 1 (*Continued*)

between 765 and 780 $cm^{-1}$ which is stronger in the *o*-isomer than in the *p*-isomer (the 773 $cm^{-1}$ band of sodium *o*-chlorobenzenediazosulphonate is in fact not detectable in the *p*-chloro-compound).

The $\nu_{14}^{\|H}$ fundamental of benzene at 1037 $cm^{-1}$ (1033 $cm^{-1}$ with the liquid; cf. Herzfeld, Ingold, and Poole 1946) is seen with all the specimens examined. (In the case of azopyridine the peak at 1036 $cm^{-1}$ is presumably due to a perpendicular bending motion of the C—C—C ring members; this in pyridine displays itself at 1037 $cm^{-1}$.) Following *p*-substitution there is a slight shift to higher frequencies (1070 $cm^{-1}$) while *o*-substitution leaves it around 1040 $cm^{-1}$.

TABLE 1

| *o*-Chlorobenzenediazosulphonates | | | | | Assignment |
|---|---|---|---|---|---|
| *trans*-Sodium ($cm^{-1}$) | *cis*-Sodium ($cm^{-1}$) | Potassium ($cm^{-1}$) | Silver ($cm^{-1}$) | Barium ($cm^{-1}$) | |
| 689 | 688 | 694 | 684 | 690 | |
| 713 | 721 | 719 | 711 | 712 | |
| | 745 | 750 | | 750 | |
| 771 | 772 | 766 | 771 | 768 | |
| 788 | 787 | 791 | 787 | 789 | *o*-Disubstituted benzene |
| 885 | 886 | 882 | 886 | 883 | |
| 962 | | 948 | 962 | 947 | C—N |
| 998 | 996 | 983 | 996 | 996 | |
| 1033 | 1033 | 1031 | 1030 | 1030 | $\nu_{14}$ of benzene |
| 1056 | 1052 | 1052 | 1056 | 1057 | $RSO_3^-$ |
| 1120 | 1104 | 1121 | 1120 | 1125 | |
| 1154 | 1143 | 1154 | 1152 | 1148 | C—N |
| 1188 | | 1191 | 1198 | 1192 | |
| 1240 | 1234 | 1239 | 1249 | 1211 | $RSO_3^-$ |
| 1272 | 1272 | 1266 | | 1258 | |
| 1300 | 1302 | 1293 | 1302 | 1296 | C—N |
| | 1339 | | | | |
| 1430 | 1430 | 1440 | 1425 | 1432 | N=N? |
| 1490 | 1485 | 1500 | 1485 | 1485 | Phenyl |
| 1575 | 1575 | 1575 | 1575 | 1575 | N=N? and phenyl |
| 1625 | 1620 | | | 1615 | Hydrated water |
| 1640 | 1635 | 1644 | | | |

| *p*-Chlorobenzenediazosulphonates | | | | Benzene Sodium Diazosulphonates | | Assignment |
|---|---|---|---|---|---|---|
| Sodium ($cm^{-1}$) | Potassium ($cm^{-1}$) | Silver ($cm^{-1}$) | Barium ($cm^{-1}$) | *o*-Iodo- ($cm^{-1}$) | *p*-Iodo- ($cm^{-1}$) | |
| 688 | 688 | 682 | 688 | 674 | 676 | |
| 719 | 721 | 720 | 721 | 706 | 708 | |
| | | | | 765 | | *o*-Disubstituted benzene |
| 780 | 775 | 778 | 779 | 783 | 778 | |
| 836 | 837 | 834 | 834 | | 833 | *p*-Disubstituted benzene |
| 893 | 890 | 894 | 890 | 884 | 892 | |
| 960 | 964 | 953 | 960 | 955 | 962 | C—N |
| 1008 | 1005 | 1007 | 1007 | | 1006 | |
| | | | | 1027 | | $\nu_{14}$ of benzene |
| 1060 | 1053 | 1038 | 1050 | 1058 | 1070 | $RSO_3^-$ |
| 1072 | 1063 | | 1076 | | | $\nu_{14}$ of benzene |
| 1090 | 1086 | 1084 | 1086 | | | |

TABLE 1 (*Continued*)

| *p*-Chlorobenzenediazosulphates | | | | Benzene Sodium Diazosulphonates | | Assignment |
|---|---|---|---|---|---|---|
| Sodium ($cm^{-1}$) | Potassium ($cm^{-1}$) | Silver ($cm^{-1}$) | Barium ($cm^{-1}$) | *o*-Iodo- ($cm^{-1}$) | *p*-Iodo- ($cm^{-1}$) | |
| 1101 | 1110 | 1102 | 1097 | | 1101 | |
| 1151 | 1149 | 1142 | 1149 | 1154 | 1154 | C—N |
| | | 1159 | | | | |
| 1188 | 1193 | 1189 | 1180 | 1188 | 1188 | |
| 1232 | 1232 | 1220 | 1241 | 1224 | 1225 | $RSO_3^-$ |
| | 1252 | | | 1266 | | |
| 1284 | 1267 | 1283 | 1279 | | | |
| 1297 | 1298 | 1300 | 1300 | 1293 | 1290 | C—N |
| 1404 | 1401 | 1400 | 1400 | 1430 | 1400 | N=N? |
| | | | 1486 | | | |
| 1504 | 1500 | 1497 | 1494 | 1495 | 1505 | Phenyl |
| 1580 | 1579 | 1580 | 1573 | 1575 | 1575 | N=N? and phenyl |
| 1588 | 1589 | 1590 | 1589 | | | |
| | 1637 | | | 1620 | | Hydrated water |
| | 1651 | 1652 | | 1630 | | |
| | 1680 | | | | | |

| Diazocarboxyamides | | | Sodium Diazosulphonates | | | Assignment |
|---|---|---|---|---|---|---|
| Benzene- ($cm^{-1}$) | *p*-Chloro-benzene- ($cm^{-1}$) | *p*-Bromo-benzene- ($cm^{-1}$) | 2,4,6-Tri-bromo-benzene ($cm^{-1}$) | 4,6-Dibromo-2-methyl-benzene ($cm^{-1}$) | *p*-Diphenyl ($cm^{-1}$) | |
| | | | 678 | | 672 | |
| 685 | 703 | 678 | 694 | 700 | 692 | |
| 739 | 722 | 725 | 721 | 721 | 722 | |
| 751 | | | 732 | 748 | | |
| 786 | 772 | 776 | 772 | 766 | 761 | |
| | | | | | 785 | |
| 843 | 839 | 828 | 836 | 818 | 834 | |
| | | | 861 | 856 | 849 | |
| 928 | | 867 | | 868 | | |
| | | | 883 | 891 | 895 | |
| 944 | 961 | 957 | | | 966 | C—N |
| 1001 | 1011 | 1039 | 981 | 1040 | 1040 | |
| 1074 | 1088 | 1073 | | 1071 | 1075 | $\nu_{14}$ of benzene |
| | 1124 | 1125 | | | | |
| 1150 | 1158 | 1186 | | 1174 | 1172 | C—N |
| 1196 | 1196 | 1201 | | 1196 | | $CONH_2$ |
| | 1244 | 1243 | | 1235 | 1232 | $RSO_3^-$ |
| | | | | 1266 | 1248 | |
| 1308 | 1300 | 1304 | | 1309 | 1298 | C—N |
| 1350 | 1344 | 1343 | | | 1336 | |
| 1430 | 1400 | 1402 | 1401 | 1392 | 1398 | N=N? |
| 1505 | 1490 | 1490 | 1492 | 1490 | 1494 | Phenyl |
| 1590 | 1578 | 1565 | 1575 | 1575 | 1575 | N=N? and phenyl |
| | | | | 1585 | | |
| | | | 1655 | 1628 | | Hydrated water |
| 1700 | 1735 | 1745 | | | | C=O |

TABLE 1 (*Continued*)

| Benzenediazotates | | | | Assignment |
|---|---|---|---|---|
| *trans*-Potassium ($cm^{-1}$) | *cis*-Potassium ($cm^{-1}$) | Sodium *p*-Nitro- ($cm^{-1}$) | Potassium *p*-Methoxy- ($cm^{-1}$) | |
| | 683 | | 669 | |
| 688 | 698 | 697 | 703 | |
| 720 | 738 | 715 | 722 | |
| 756 | 760 | 753 | 765 | |
| 832 | 843 | | 833 | |
| | | 855 | 857 | |
| 886 | 889 | | 891 | |
| 904 | | | | |
| 917 | 911 | 920 | 917 | C—N |
| | | | 973 | |
| | | | 1008 | $C_6H_5$.OMe |
| | | | 1035 | |
| 1073 | 1070 | | | |
| 1102 | | 1106 | 1110 | |
| 1124 | | | | |
| 1156 | 1146 | | 1152 | C—N |
| 1168 | 1166 | 1186 | 1178 | |
| | 1195 | | | |
| 1222 | 1234 | 1245 | 1248 | |
| | | 1284 | | |
| 1306 | 1304 | 1310 | 1296 | C—N |
| 1324 | 1338 | 1330 | 1340 | } Aromatic nitro- |
| | | 1350 | | |
| 1398 | 1405 | 1400 | 1400 | N=N? |
| | 1445 | | | |
| | 1465 | | | |
| 1485 | 1485 | 1505 | 1505 | Phenyl |
| 1575 | 1580 | 1585 | 1580 | } Phenyl and N=N? |
| | | 1595 | | |
| 1670 | 1670 | 1680 | 1635 | |

| *p*-Chlorobenzene-diazosulphone ($cm^{-1}$) | Phenyl *p*-Tolylazoxysulphones | | | |
|---|---|---|---|---|
| | Unsubstituted ($cm^{-1}$) | *p*-Dimethylamino- ($cm^{-1}$) | *p*-Diethylamino- ($cm^{-1}$) | |
| 681 | 695 | 689 | 693 | |
| 716 | | | 721 | |
| 746 | 764 | 739D | 738 | |
| 776 | 780 | 787 | 776 | |
| | 799 | 801 | 801 | |
| | 812 | 815 | 814 | |
| 826 | | 826 | 826 | *p* - Disubstituted benzene |
| 839 | 837 | 838 | 840 | |

TABLE 1 (*Continued*)

| *p*-Chlorobenzene-diazosulphone ($cm^{-1}$) | Phenyl *p*-Tolylazoxysulphones | | | Assignment |
|---|---|---|---|---|
| | Unsubstituted ($cm^{-1}$) | *p*-Dimethylamino- ($cm^{-1}$) | *p*-Diethylamino- ($cm^{-1}$) | |
| 880 | 897 | 943 | 944 | C—N |
| 1006 | 1018 | 1015 | 1004 | |
| 1084 | 1085 | 1082 | 1080 | $\nu_{14}$ of benzene |
| 1139 | | 1125D | 1125D | C—N |
| 1163 | 1168 | 1151 | 1162 | —$SO_2$— |
| 1180 | 1188 | 1185D | 1189D | |
| | | 1265 | 1266 | Azoxy-group |
| 1306 | 1296 | 1312 | 1312 | C—N |
| | | 1328 | 1332 | $C_6H_5.NR_2$ |
| 1350 | 1352 | 1350 | 1352 | —$SO_2$— |
| 1400 | 1395 | 1398 | 1398 | N=N? |
| 1445 | 1428 | | 1475 | |
| 1480 | 1475 | 1488 | 1488 | Phenyl and azoxy-group |
| | | 1524 | 1515 | |
| 1565 | 1580 | 1580 | 1580 | N=N? and phenyl group |
| | 1598 | 1590 | 1602 | |

| Azobenzenes | | Benzenediazocyanides | | 2,2′-Azo-pyridine ($cm^{-1}$) | 1-Methyl-1,2,3-benzo-triazole ($cm^{-1}$) | Assignment |
|---|---|---|---|---|---|---|
| *trans*- ($cm^{-1}$) | *cis*- ($cm^{-1}$) | *o*-Chloro- ($cm^{-1}$) | *p*-Chloro- ($cm^{-1}$) | | | |
| 689 | 690 | 713 | 712 | | | |
| | 698 | | | | | |
| | 755 | | | 736 | 739 | |
| 773 | 774 | 773 | | 795 | 770 | |
| | 836 | | | | | |
| 851 | 843 | | 841 | | | *p* - Disubstituted benzene |
| | 867 | | | | | |
| 923 | 918 | | | | 936 | C—N |
| | 967 | 965 | | 960 | | |
| 982 | 987 | | 992 | | | |
| 998 | 998 | 999 | 1010 | 988 | 994 | |
| 1019 | 1023 | 1031 | 1029 | 1036 | 1024 | |
| 1070 | 1066 | 1065 | 1067 | | | $\nu_{14}$ of benzene |
| | | | 1089 | 1096 | 1110 | |
| 1150 | 1150 | 1162 | 1154 | 1136 | 1120 | C—N |
| | 1178 | | | | 1164 | |
| 1220 | | 1208 | 1210 | 1218 | 1196 | |
| | | 1256 | | 1260 | 1274 | |
| 1297 | | 1308 | 1308 | | 1296 | C—N |
| 1395 | 1430 | 1390 | 1385 | 1420 | 1415 | N=N? |
| | | | 1420 | | | |
| 1485 | 1470 | | 1465 | | | |
| | 1508 | | | | 1495 | Phenyl |
| 1575 | 1575 | 1560 | 1560 | 1582 | 1587 | N=N? and phenyl |
| | | 1580 | | | 1615 | |
| | | 2160 | 2160 | | | C≡N |

TABLE 1 (*Continued*)

| 3,3-Dimethyltriazens | | | | | | |
|---|---|---|---|---|---|---|
| 1-*m*-Nitrophenyl- ($cm^{-1}$) | 1-*p*-Nitrophenyl- ($cm^{-1}$) | 1-*p*-Chlorophenyl- ($cm^{-1}$) | 1-*p*-Diphenylyl- ($cm^{-1}$) | 1-β-Naphthyl- ($cm^{-1}$) | 1-*o*-Carboxyphenyl- ($cm^{-1}$) | Assignment |
| 671 | | | | | | |
| 693 | 694 | 702 | 687 | | 691 | |
| | | | 722 | | | |
| 741 | 752 | | | 747 | | |
| | | | 761 | 769 | 760 | |
| 818 | | 812 | | 826 | 838 | |
| | 850 | 836 | 843 | 862 | | *p* - Disubstituted benzene |
| 888 | | 910 | | 886 | 909 | |
| | | | | 950 | 962 | |
| 995 | | 1003 | | 1018 | | |
| 1046 | 1042 | 1045 | 1050 | 1050 | 1036 | |
| 1078 | | 1080 | 1088 | 1086 | 1089 | $\nu_{14}$ of benzene |
| 1100 | 1096 | | 1116 | 1116 | 1116 | |
| | | | | 1142 | | |
| 1158 | 1166 | 1152 | 1162 | 1156 | 1166 | C—N |
| 1200 | 1212 | 1198 | 1228 | 1192 | | |
| | | | | 1208 | | |
| | | | | 1248 | 1240 | |
| 1270 | | 1275 | 1266 | 1280 | | |
| 1304 | 1300 | 1316 | 1300 | 1306 | 1316 | C—N |
| 1344 | 1328 | 1338 | 1346 | 1351 | 1364 | $—NMe_2$ |
| | 1364 | | | | 1387 | N—O |
| 1399 | 1404 | 1396 | 1388 | 1402 | 1402 | N=N? |
| | | 1413 | 1413 | | | |
| | 1500 | | | | 1495 | Phenyl |
| 1575 | 1588 | 1575 | 1595 | 1590 | 1575 | N=N? and phenyl |
| 1625 | | | | 1620 | 1600 | |
| | | | | | 1705 | —COOH |

| Diazoaminobenzene ($cm^{-1}$) | Silver Complex of 4,4′-Dimethyldiazoaminobenzene ($cm^{-1}$) | *p*-Aminoazobenzene ($cm^{-1}$) | *p*-Aminoazobenzene Hydrochloride ($cm^{-1}$) | Benzeneazo-β-naphthol ($cm^{-1}$) | Copper Complex of Benzeneazo-β-naphthol ($cm^{-1}$) | |
|---|---|---|---|---|---|---|
| 682 | 707 | 689 | 683 | 682 | 689 | |
| | | | 705 | | 736 | |
| 746 | | | 748 | 750 | 752 | |
| 761 | 763 | 769 | | 779 | 763 | |
| | 808 | | 799 | | 816 | |
| | 816 | | 810 | | | |

TABLE 1 (*Continued*)

| Diazo-amino-benzene (cm⁻¹) | Silver Complex of 4,4′-Dimethyl-diazoamino-benzene (cm⁻¹) | *p*-Amino-azobenzene (cm⁻¹) | *p*-Aminoazo-benzene Hydro-chloride (cm⁻¹) | Benzene-azo-β-naphthol (cm⁻¹) | Copper Complex of Benzeneazo-β-naphthol (cm⁻¹) | Assignment |
|---|---|---|---|---|---|---|
| | 827 | 835 | 825 | 829 | 837 | *p* - Disubstituted benzene |
| | | | 852 | 844 | 861 | |
| 888 | | | 890 | 871 | | |
| 900 | | | 906 | 898 | 904 | |
| | 927 | | | | 935 | |
| | 955 | | 982 | 982 | 954 | C—N |
| | | | | | 993 | |
| 1068 | 1056 | 1068 | 1080 | 1072 | 1076 | $\nu_{14}$ of benzene |
| | 1107 | 1120 | | | | |
| 1142 | | 1139 | 1138 | 1142 | 1146 | C—N |
| 1159 | 1165 | | | | 1166 | |
| 1170 | | | 1171 | 1180 | 1184 | |
| 1190 | 1214 | | | | | |
| | | 1232 | | 1248 | 1220 | |
| 1252 | 1284 | | 1262 | 1268 | 1252 | |
| 1302 | | 1304 | 1318 | 1320 | 1296 | C—N |
| 1320 | 1358 | | | 1386 | 1352 | |
| 1400 | 1408 | 1412 | 1400 | 1416 | 1402 | N=N? |
| 1432 | | | | | 1435 | |
| 1500 | 1498 | 1503 | | 1495 | 1500 | Phenyl |
| 1520 | | | {1525 | 1552 | 1544 | |
| | | | 1542 | | | |
| 1595 | 1575 | 1590 | 1595 | 1590 | 1595 | N=N? |
| 1615 | 1602 | 1615 | 1615 | 1615 | 1615 | Phenyl |
| | | | 1654 | | | |

Other well-known " phenyl " absorptions at about 1500 and 1600 cm$^{-1}$ (due to " in plane " skeletal vibrations) may also be recognized in Table 1.

Assignments for the C—N band have, in the past, been variously made: Thompson (1948) quotes 1250–1325 cm$^{-1}$ when the carbon is unsaturated or aromatic, Colthup (1950) lists 900–1300 cm$^{-1}$; workers who have studied azomethane or azobenzene indicate two regions, close to 900 and 1100–1150 cm$^{-1}$ (West and Killingsworth 1938; Imanishi and Kanda 1949; Stammreich 1950; Tetlow 1950).* Accordingly in Table 1 those frequencies occurring around the points 900, 1150, and 1300 cm$^{-1}$ are associated with C—N.

* Since this paper was submitted a note by Thomas (1953) has come to hand. Thomas accepts that in azomethane the C—N bands are associated with frequencies of 1110 cm$^{-1}$ ($\nu_a$) and 922 cm$^{-1}$ ($\nu_s$) but proceeds to point out the difficulty of attributing a well-defined frequency to a given single bond; in particular he mentions that a feature at 1327 cm$^{-1}$ in HNCO may be a $\nu_{NC}$. (R. J. W. Le F., July 30, 1953.)

Only slight differences are observed among the series of spectra of salts of *o*- and *p*-chlorobenzenediazosulphonates, although the frequency due to the sulphonate ion group apparently tends to decrease as the size of the cation increases. Assignments for $RSO_3^-$ and $R_2SO_2$ may be reasonably made to the strongest vibrations in the small regions where they are supposed to occur (cf. Colthup 1950) especially as other bands in these localities are fairly weak. The annotations " azoxy-group " are tentative and follow Langley, Lythgoe, and Rayner (1952). Effects from $NO_2$ in aromatic molecules have recently been discussed by Randle and Whiffen (1952).

### (*b*) *Isomeric Pairs of Diazo-Compounds*

Table 1 contains data for three isomeric pairs : the *cis*- and *trans*-sodium *o*-chlorobenzenediazosulphonates, the stable and labile forms of potassium benzenediazotate, and the two azobenzenes.

With the diazosulphonates, since decomposition seemed to occur whenever complete dehydration of the *cis*-sodium salt was attempted, the spectrum had necessarily to be recorded on a not completely dried sample. The spectra of the *cis*- and *trans*-salts show certain differences, the former having bands peculiar to itself at 745 (strong), 1104 (strong), and 1339 $cm^{-1}$ (medium), the latter at 962 (medium), 1120 (medium), and 1188 $cm^{-1}$ (strong).

Among the two diazotates the following characteristic absorptions appear : in the *cis*-variety, 738 (very strong), 1195 (strong), 1338 (strong), 1445 (weak), 1465 $cm^{-1}$ (medium), and in the *trans*-isomer, 720 (medium), 1102 (weak), 1124 (medium), 1324 $cm^{-1}$ (weak).

Tetlow (1950), between 600 and 1400 $cm^{-1}$ similarly noted that *cis*-azobenzene gave unique bands at 755 (strong), 970 (weak), and 1157 $cm^{-1}$ (medium) in contrast to *trans*-azobenzene which gave them at 1225 (medium) and 1300 $cm^{-1}$ (medium). The present work confirms all but the third of these features (our comparable readings being 755, 967, 1178 $cm^{-1}$ for the *cis*- and 1220 and 1297 $cm^{-1}$ for the *trans*-forms respectively) and adds 836 (weak), 867 (weak), 1430 (weak), 1470 (strong), and 1508 $cm^{-1}$ (medium) for the *cis*-isomer, and 1395 (weak) and 1485 $cm^{-1}$ (medium) for the *trans*-isomer.

For the three isomeric pairs examined, we note the emergence in the *cis*-forms only of absorptions between 720 and 760 $cm^{-1}$ ; these absorptions, being intense, should have obvious applications in the analysis of *cis-trans*-mixtures.

The above remarks concern differences of more than 12 $cm^{-1}$. An inspection of the curves will show, however, that the contrasts are not more extensive than those between the sodium *o-trans*- and *p-trans*-chlorobenzenediazosulphonates, nor are they more marked than others found for *cis-trans*-isomers in C=C containing substances (cf., for example, Brackman and Plesch (1952) on the stilbenes).

### (*c*) *Frequencies Associated with the* $-N{=}N-$ *Link*

A survey of the observations listed in Table 1 reveals that there are common frequencies in the usual " double bond " region around 1400 and 1580 $cm^{-1}$, the latter being, in most cases, doublets. The same two features are to be seen

in earlier infra-red spectra. They occur among the diazocyanides studied by Anderson, Le Fèvre, and Savage (1947). The curves published by Barnes, Liddel and Williams (1943) show a weak absorption for azobenzene at *c*. 1400 $cm^{-1}$ and a stronger one at *c*. 1590 $cm^{-1}$. The last-named authors also include a spectrum of pyridazine, an N=N containing structure, displaying strong absorptions at 1405 and 1575 $cm^{-1}$.

Two recordings of Raman displacements with azobenzene are extant but do not agree with one another : Imanishi and Kanda (1949), using the yellow mercury line as exciter and azobenzene dissolved in methanol or carbon disulphide, reported shifts at 1138, 1433, and 2636 $cm^{-1}$ only ; Stammreich (1950), using 5875·6 Å helium light and carbon tetrachloride, observed (in addition to four at frequencies below 870 $cm^{-1}$) the $\Delta\nu$ listed in Table 2 against possibly

TABLE 2

| $\Delta\nu$ ($cm^{-1}$) and Strengths (Stammreich) | Azobenzene | |
|---|---|---|
| | *trans*- ($cm^{-1}$) | *cis*- ($cm^{-1}$) |
| 870 (0·5) | — | 867 (2) |
| 1002 (4) | 998 (3) | 998 (1) |
| 1152 (10) | 1150 (4) | 1150 (2) |
| 1184 (4) | — | 1178 (2) |
| 1400 (3) | 1395 (1·5) | — |
| 1442 (8) | — | 1430 (3) |
| 1487 (4) | 1485 (5) | 1470 (8)<br>1508 (6) |
| 1601 (5*b*) | — | — |

comparable points from our Table 1. Although Stammreich annotates the frequency at 1400 $cm^{-1}$ as " nicht ganz sicher ", and the possibility of an infra-red Raman coincidence at this point remains open, it appears that the 1575 $cm^{-1}$ absorption bands of the two azobenzenes are not reproduced in the Raman spectrum.

Even so, there are other definite coincidences, from which we should conclude that *trans*-azobenzene does not have a centre of symmetry. Yet the published X-ray analysis (de Lange, Robertson, and Woodward 1939) provides no firm evidence of distortions which would deprive the molecular skeleton of such a centre. The apparent contradiction may be due to a breaking-down for the crystalline or liquid states of those selection rules which depend upon a strict preservation of the symmetry of the equilibrium configuration (cf. Herzfeld (1946) and associated papers by Ingold and collaborators on benzene).

Azomethane shows non-coincidence at 1575 $cm^{-1}$ but in the reverse sense to azobenzene. West and Killingsworth (1938) found the former to have frequencies in its Raman spectrum of 1575 and 1442 $cm^{-1}$ (Kahovec *et al.* (1938) give 1573 and 1428 $cm^{-1}$ for these shifts) corresponding—at first sight—to only

one, at 1430 $cm^{-1}$, in the infra-red spectrum. West and Killingsworth consider the nearness of 1442 and 1430 $cm^{-1}$ an accidental coincidence and relate the former to a symmetrical N=N stretching mode and the latter to antisymmetric bending vibrations of the H atoms in the methyl groups. Herzberg (1945) indicates a different assignment and lists the Raman displacement of 1576 $cm^{-1}$ as $\nu_{N=N}$. Its absence in absorption is understandable only if the methyl groups have certain special orientations to the $C-N_2-C$ plane. Fulfilment of these conditions seems unlikely, and we feel that azomethane should be reinvestigated.

In an attempt to check Herzberg's assignment we have made a number of estimates via the empirical relation of Gordy (1946), namely:

$$k=\frac{aN}{d^2}(\chi_A\chi_B)^{3/4}+b,$$

where $k$ is the stretching force constant multiplied by $10^{-5}$ dyn/cm, $\chi_A$ and $\chi_B$ are the electronegativities of the terminal atoms of the bond in question, $d$ is the internuclear distance, and $N$ is the bond order; $a$ and $b$ are constants for certain broad classes of molecules (they have values 1·67 and 0·30 respectively in the present application). The electronegativity of nitrogen is taken as 2·98 following Gordy (an earlier estimate by Pauling (1944) was 3·00). The N=N internuclear separation $d$ probably does not vary much from one azo-compound to another, for it appears as 1·23 Å from the X-ray analysis of azobenzene (de Lange, Robertson, and Woodward 1939), as $1{\cdot}25\pm0{\cdot}04$ Å in F—N=N—F, and as $1{\cdot}24\pm0{\cdot}05$ Å in azomethane (cf. for references Allen and Sutton 1950).

This near-constancy of $d$ suggests a similar near-constancy of nitrogen-nitrogen bond orders throughout azo-compounds, since it seems unlikely that nitrogen-nitrogen links do not follow at least roughly the sort of generalizations that apply to the various multiplicities of carbon-carbon, carbon-nitrogen, and carbon-oxygen links (cf. Beach, Brockway, and Pauling 1935; Pauling 1944). For the last three cases Gordy (1947) has found that $N$ can be satisfactorily expressed by the equation

$$N=ad^{-2}+b,$$

(cf. also Bernstein 1947). Now, in azomethane resonance (between structures such as R—N=N—R, $R^-$ : N—N · $R^+$, etc.) cannot be imagined as significantly affecting the multiplicity of the nitrogen-nitrogen link; accordingly taking $N=2$ in Gordy's relation, $k$ emerges as $12{\cdot}6\times10^5$ dyn/cm, which inserted in the equation for a simple harmonic oscillator, namely

$$\nu=1307\ (k/\mu)^{\frac{1}{2}}\ cm^{-1},$$

where $\mu$ is the reduced mass, yields a frequency of about 1770 $cm^{-1}$. From what has been said above regarding the closeness of $d_{N=N}$ for the three molecules R—N : N—R in which the R's are either Me—, F—, or $C_6H_6$— (i.e. are groups having widely differing conjugating powers towards unsaturated centres to which they are attached, cf. literature summarized by Le Fèvre 1953), much the same $\nu_{N:N}$ should be computed for azobenzene as for azomethane.

Such forecasts are some 200 $cm^{-1}$ higher than the $\nu_{N=N}$ allotted in azomethane by Herzberg or either of the common frequencies now found among our azo-compounds. Two possibilities are open: (*a*) that absorption due to $-N=N-$ is weak and we have missed it, or (*b*) that Gordy's rule, although excellent for the 71 examples quoted in his paper (including cases involving $C-N$, $C\equiv N$, and the nitrogen molecule) is invalid for $N=N$. With present equipment we cannot examine (*a*) further than we already have done, but *apropos* (*b*) we note that Gordy himself finds $N-N$ in hydrazine to be exceptional. We have therefore used also the equations of Badger (1934, 1935), Mecke (1950), and Guggenheimer (1950) with the following results:

$$\nu_{N:N} \text{ via Badger's relation} = 1730 \text{ cm}^{-1},$$
$$\nu_{N:N} \text{ via Mecke's relation} = 1660 \text{ cm}^{-1},$$
$$\nu_{N:N} \text{ via Guggenheimer's relation} = 1420 \text{ cm}^{-1}.$$

The first of these approaches in order the value obtained via Gordy's relationship. The second involves the mean atomic refraction of the two nitrogen atoms—a quantity which for the azo-group cannot be stated accurately (Anderson, Le Fèvre, and Wilson 1949). The third,

$$k = 2\cdot738\cdot10^5(Z_1Z_2)^{\frac{1}{2}}d_{\text{equilib.}}^{-2\cdot46},$$

requires only the number ($Z_1$) of electrons on each nitrogen atom, so that $Z_1Z_2$ can, in contrast to the variables entering the other equations, be written with certainty as $5\times5$.

It is possible that the two common absorptions under attention do not arise from the $-N_2-$ group at all. As already mentioned, many of the bands at 1580 $cm^{-1}$ are recorded as doublets: a splitting (to 1585 and 1606 $cm^{-1}$) of the unperturbed fundamental frequency, 1596 $cm^{-1}$, is reported in the Raman spectrum of benzene by Herzfeld *et al.* (1946), while *liquid* benzene shows infra-red bands at 1585 and 1604 $cm^{-1}$. Features in this region, due to $C-C$ ring stretching vibrations, are usually seen in phenyl-derivatives; they occur also in pyridine (at 1600 $cm^{-1}$, Turkevich and Stevenson 1943) and among other heterocycles (e.g. pyrimidines, Brownlie 1950). Further, since *liquid* benzene shows a band at 1381 $cm^{-1}$ and various aromatic compounds display absorption near this point [e.g. 1422 $cm^{-1}$ in solid cinnamic acid (Randall *et al.* 1949) *cis*- and *trans*-stilbenes at 1413 and 1391 $cm^{-1}$ respectively (Brackman and Plesch 1952)] while *liquid* pyridine does so at 1439 $cm^{-1}$, and the pyrimidines between 1400 and 1420 $cm^{-1}$, clearly we cannot be certain that the frequencies now observed around 1400 $cm^{-1}$ are not due to the same types of skeletal vibrations which produce the 1381 $cm^{-1}$ band in benzene.

## V. Conclusions

From the above discussion it is evident that the unknown $\nu_{\text{stretching}}^{N=N}$ is either (*a*) not active in infra-red absorption, or (*b*) displays itself in coincidence with other effects arising from vibrations—which could occur in all our compounds—of the aromatic or heterocyclic skeletons to which the $-N=N-$ unit is attached. Since we have examined *solid* specimens we do not expect (*a*) to be true.

Regarding (*b*), because no other common band likely to be due to a double bond has emerged, we infer that —N=N— stretching motions cannot be affecting the spectra elsewhere than in the neighbourhoods of the points 1406 and 1579 cm$^{-1}$. Which, if either, of these is $\nu^{N=N}_{\text{stretching}}$ is impossible to say at the present stage, but in the light of the data for azomethane the lower value seems slightly to be preferred ; such a choice is contrary to that made by Herzberg (1945).

## VI. Acknowledgments

We wish to record our gratitude to Dr. E. C. Gyarfas for preparing the metallic derivatives of benzeneazo-β-naphthol and diazoaminobenzene, and to Messrs. W. Hermon Slade & Co. Pty. Ltd. for a donation which has assisted our general programme on azo-compounds of which this paper is a part.

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# STUDIES IN RELATION TO BIOSYNTHESIS

## I. SOME POSSIBLE ROUTES TO DERIVATIVES OF ORCINOL AND PHLOROGLUCINOL

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[*Manuscript received May 28, 1953*]

*Summary*

Correlations of the structures of some natural products containing orcinol or phloroglucinol nuclei, and related compounds, support the hypothesis that the molecules are elaborated, at least in part, by the head-to-tail linkage of acetate units. Chemical and biochemical evidence is cited in support of the hypothesis.

### I. INTRODUCTION

The isoprene rule is an outstanding example of the usefulness of considering biogenetic relations in natural products. In the present state of biochemical knowledge it is often difficult to know exactly what realities such structure correlations represent; nevertheless, the generalizations are not of speculative interest only. They draw attention to previously unsuspected relations between classes of substances; they are useful in structure determinations to limit the number of possible formulae; they often suggest laboratory methods of synthesis, and they may assist the more fundamental biochemical approach by indicating possible lines of investigation. Work on the biogenetic relations of alkaloids shows how fruitful such theories can be.

The structural units in isoprenoid compounds are marked out by carbon atoms, and in alkaloids by carbon and nitrogen atoms, and are remarkably persistent because of the stability of the molecular skeleton. They are therefore usually easy to distinguish and may be quite large. Their size means, however, that some of the more fundamental processes in biosynthesis cannot be illuminated by their study; no information is, for example, available from such work as to how the isoprene units arise. Units which should be marked out by oxygen atoms attached to the skeleton may not be easily seen because of the ease of occurrence of biological oxidation-reduction reactions. It is known from work using radioactive acetic acid (e.g. Little and Bloch 1950) that fatty acids and steroids are built up from acetate units, but the almost complete loss of oxygen renders impossible detection of the units merely by inspecting the formulae. However, in some natural products containing a higher proportion of oxygen the formulae appear to indicate that the molecules are formed wholly or in part by the head-to-tail linkage of acetate units. The present paper proposes to survey some of the structural evidence. In order to establish such an hypothesis with some degree of probability series of related compounds must

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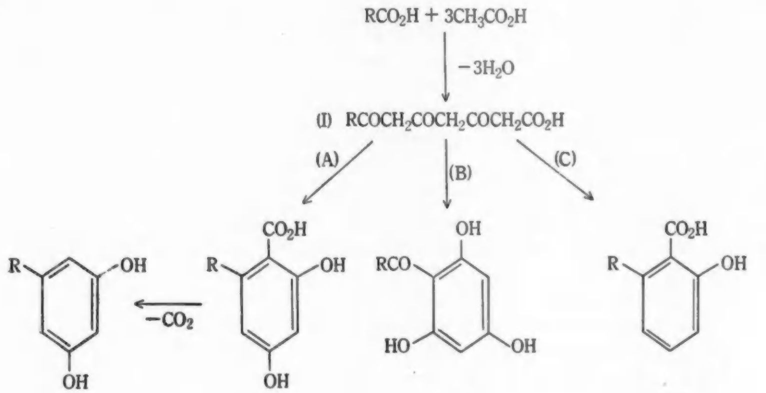
be considered in order to find common structural factors; also required is the admission that biological reactions may be of well-known laboratory types such as aldol-condensation, C-acylation, reduction, dehydration, methylation, and oxidation. In a remarkable paper more than 45 years ago, Collie (1907) advanced similar views, but it now appears that he tried to push them too far.

The head-to-tail linkage of acetate units could lead to phenolic substances in several ways. Continuation of the known process of elaboration of a fatty acid $RCO_2H$ (R contains an odd number of carbon atoms) by the addition of unreduced acetate units could lead to a polyketone of types I or VI, which by ring closure through aldol condensation or C-acylation could give rise to orcinol or phloroglucinol derivatives respectively, as shown below. Decarboxylation of I or II (known to be a facile process) could produce III, while reduction of a carbonyl group not involved in the cyclization to an hydroxyl group could give rise to V by dehydration. Cyclization of VI, containing one more intact acetate unit, could lead to a molecule containing a carbonyl group in the β-position of the side chain (VII) by aldol-condensation, and by C-acylation to the diketone (VIII). Many examples of the types II, III, IV, and VII are found in nature and the scheme can be further extended; for example, the acid $RCO_2H$ may be of a type other than a fatty acid.

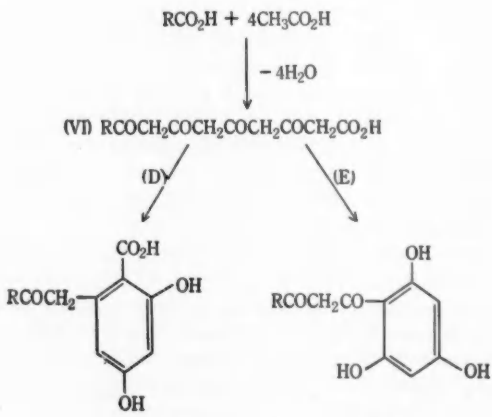
The co-occurrence of compounds representing different branches of the above scheme may be considered significant if it is frequent enough. The heartwoods of nearly 100 species of pines are known to contain in association two groups of compounds, one based on pinosylvin (IX), the other on 5,7-dihydroxyflavone (X), individual components differing in degree of methylation and oxidation level (Lindstedt 1951). These two groups could be derived by the routes A and B from common precursors (I, R, PhCh=CH–, or closely related compounds), that is, by the addition of three acetate units to cinnamic acid or related compounds. Such acids would presumably be related biologically to phenylalanine, which is probably derived from shikimic acid (XI) (Davis 1951), and hence from carbohydrate. Only the phloroglucinol or orcinol rings of X or IX would thus come by any direct route from acetate units. Other anthoxanthins and anthocyanins could arise in the same way. The evidence recently surveyed by Geissman and Hinreiner (1951) does not contradict such an hypothesis.

A recent attempt to test the theory by examining the biogenesis of quercetin (3,3′,4′,5,7-pentahydroxyflavone) (Birch, Donovan, and Moewus, unpublished data) has confirmed that in *Chlamydomonas eugametos* phenylalanine does in fact play the expected rôle. The phloroglucinol ring appears in this case to be derived from phloroglucinol itself, in agreement with older theories (e.g. Robinson 1948). If this method is a general one it raises considerable difficulties, not encountered in the acetate hypothesis. For example, it has been shown that butein (3′,4′,7-trihydroxychlacone, using flavonoid numbering) and cyanidin (a 3,3′,4′,5,7-pentahydroxyflavylium salt) arise in *Dahlia variabilis* Desf. from a common precursor (cf. Price and Lawrence 1940). In the production of butein it would be necessary on the phloroglucinol hypothesis to remove reductively a hydroxyl group from the phloroglucinol ring: a most unlikely event. On the

acetate hypothesis a reduction stage could precede formation of the ring. Introduction of further hydroxyl groups should present no biological difficulties (Seshadri 1948, 1950). Further experimental work is in progress on these problems and will be reported elsewhere.




(III) (II) (IV) (V)

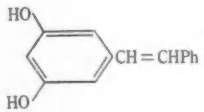



(VII) (VIII)

The acetate hypothesis suggests some relationships in this series which are not immediately obvious. For example, yangonin (XII) and its analogues methysticin and kawain may be derived by a variation of the flavone process which stops after the addition of two acetate units, cyclization then occurring on oxygen rather than carbon. Cotoin (XIII) and phenylcoumalin (XIV) occur

together in coto-bark and could be derived as the result of the addition of two and one acetate units respectively to benzoylacetic acid, or of three and two acetate units to benzoic acid.*

Eugenone (XV) and the 2-methyl-5,7-dihydroxybenzochromone derivatives eugenin, eugenitol, and angustifolionol may be examples of compounds corresponding to route E. It is interesting that the last three compounds contain in the phloroglucinol ring, no methyl, one, and two methyl groups, so such substituents apparently are adventitiously introduced and can be neglected in considering the genesis of the main skeleton.



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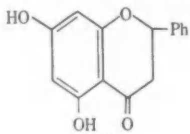

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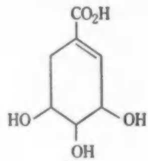

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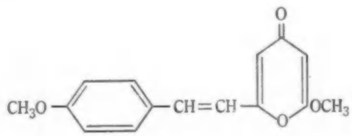

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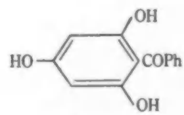

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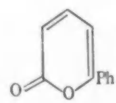

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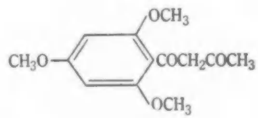


(XV)

The formulae of a large number of depsides are derivable according to schemes A, C, and D. These molecules contain units of the type II ($R = CH_3$, $n-C_3H_7$, $n-C_5H_{11}$) or VII ($R = n-C_5H_{11}$) sometimes both in the same molecule as in olivetoric acid (XVI). Other depsides with more condensed nuclei could be derived by further processes from the same units, for example, physodic acid (XVII) and didymic acid (XVIII). Some mould metabolic products of similar types are known, for example, XIX (Oxford and Raistrick 1933; Raistrick and Stickings 1951).

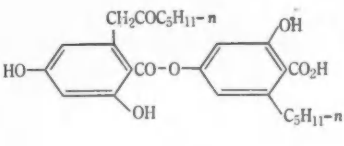
* It would not be possible at this stage to distinguish between a route beginning with a $C_6$–$C_3$ compound and a $C_6$–$C_1$ compound: they might in fact be identical. It has been shown with one organism at least (Baddiley *et al.* 1950) that the last two carbon atoms of the side chain of tyrosine might well be derived almost directly from an acetate unit.

Related to this series are a number of phenols carrying a long side chain ($n-C_{15}$ or $n-C_{17}$) (Backer and Haack 1941 ; Izzo and Dawson 1950), which have evident affinities with the relatives of palmitic and stearic acids. The formulae often lack an expected carboxyl or hydroxyl group, but it seems significant that anacardic acid (XX) and cardol (XXI) occur together, as do ginkgolic acid and bilobol which have two more hydrogen atoms in the side chain. Pelandjuaic acid is similar to anacardic acid, except that it carries a $n-C_{17}H_{31}$ side chain. A number of related compounds contain the unusual *o*-alkylcatechol structure (XXII) ($R=n-C_{15}H_{27}$, $n-C_{15}H_{29}$, $n-C_{17}H_{31}$, $n-C_{17}H_{33}$) and may arise by an oxidative decarboxylation of compounds of the type XX. That some compounds of this series are mixtures containing side chains of varying degrees of unsaturation with double bonds which may not necessarily be in the positions usually found in fatty acids seems to indicate that they are built up by processes parallel rather than sequential to the formation of such acids. In campnospermonol (XXIII), however, an oleyl residue and an extra acetate unit are clearly visible.

Among many other compounds which can be fitted into schemes based on acetate units, an attractive example is griseofulvin (XXIV) which could arise from the folding of a straight chain of seven units as shown in XXV. The same mould produces 6-methylsalicylic acid, which is an example of scheme (C). Many coumarins contain oxygenated nuclei which might be formed in the manner here described. However, they could also originate from the cinnamic acids by an oxidative ring closure and no evidence is available to distinguish between the possibilities.

If other units known to be involved in biochemical processes, such as pyruvate and oxalacetate, are employed as building units the range of compounds for which a schematic biogenesis can be suggested is further extended. Examples derivable from pyruvate are nephrosteric and protolichesteric acids (XXVI, $R=n-C_{11}H_{23}$, $n-C_{13}H_{27}$) and from oxalacetate the monomethyl ester of XXVII (caperatic acid). The use of formaldehyde or a similar $C_1$ unit, as in the alkaloid series, would permit further extension of the scope of such schemes.

In some cases subsequent oxidation may obscure the original scheme, but the formulae of a number of hydroxybenzoquinones and hydroxyanthraquinones are at least consistent with the production of the skeleton from acetate units. The quinones embelin and rapanone (XXVIII, $R=n-C_{11}H_{23}$, $n-C_{13}H_{27}$) could arise from compounds of type II or III. An exception is mesaquinone (XXVIII, $R= -n-C_{20}H_{39}$) (Hiramoto 1939) with an even number of carbons in the side chain. In view of the difficulty of determining the exact size of such side chains this compound might well be reinvestigated. The anthraquinones (XXIX) and solorinic acid (XXX) may be formed as shown, by way of the anthrones which are known often to accompany such quinones. However, although many natural anthraquinones fit readily into such schemes others do not. Their genesis may be different in type, or extensive oxidation-reduction may occur. Compounds containing the $CH_2OH$ side chain are common, and it is not possible to decide whether they may arise from a $CH_3$ or a $CO_2H$ of acetate.



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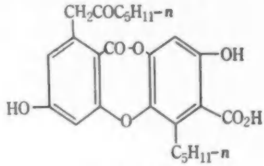

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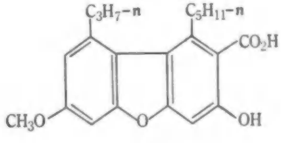

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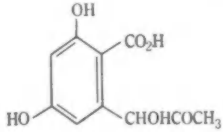

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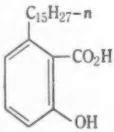

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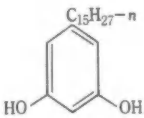

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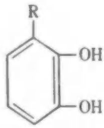

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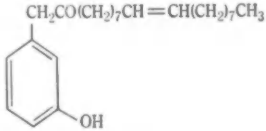

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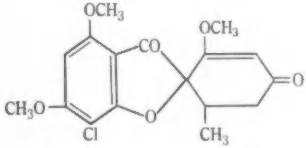

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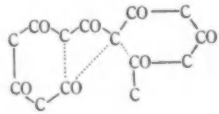

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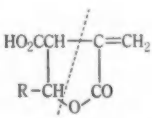

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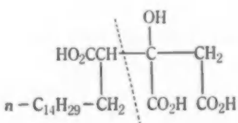
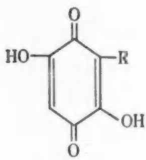


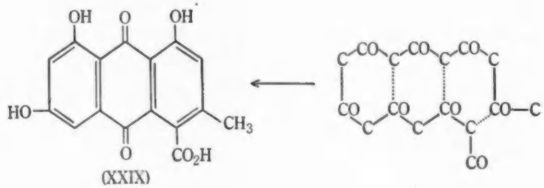
(XXVII)

Furthermore, it is known that the biological reduction of carboxyl to methyl occurs with ease in some cases, for example, in the formation of *iso*leucine from acetate units (Umbarger and Adelberg 1951). Consideration of such cases will

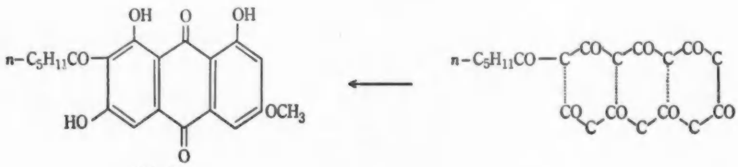
have to be postponed until the facts are clear with simpler examples. A superficially different scheme for the biogenesis of the anthraquinone emodin has been put forward by Mühlemann (1949), but close examination shows that this also involves the junction of C—CO units.



(XXVIII)



(XXIX)

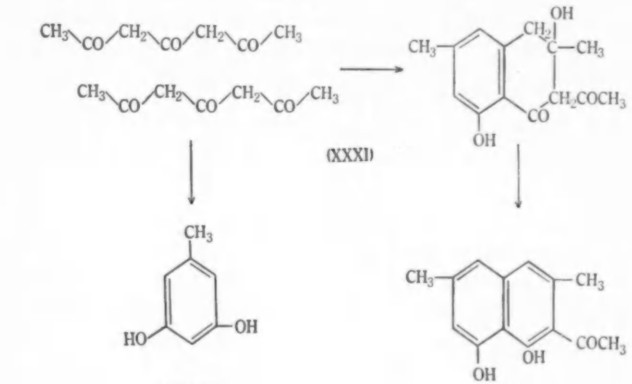


(XXX)

In order that speculations of the type above should have a firm basis, two conditions must be fulfilled. One is that reactivity of the kind postulated must be in accord with laboratory experience, and the other is that some biochemical analogies should be available. In most cases exact laboratory catalysts and conditions obviously could not occur in living matter. However, a laboratory analogy should not necessarily be considered invalid for this reason. In enzyme-catalysed reactions the correct types of activation may be achieved by more subtle, but nevertheless fundamentally similar means when considered from the view point of reaction mechanisms. The replacement of oxygen by sulphur, considered below, is a known example of how esters are activated for biological acylations, thus rendering unnecessary powerful catalysts in the reactions.

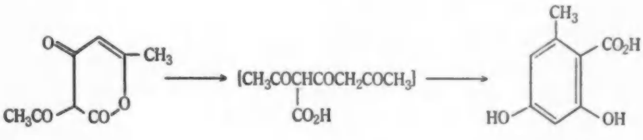
There is considerable laboratory evidence that β-polyketones can be cyclized to phenols. Collie (1893, 1907) found that diacetylacetone (XXXI) cyclizes under strongly alkaline conditions to orcinol (XXXII) and under weakly alkaline conditions in the stages shown to form XXXIII. Collie and Myers (1893) and Collie (1907) also observed the formation by alkaline treatment of dehydracetic acid (XXXIV) of a poor yield of orcinol and an orcinolcarboxylic acid, at first stated not to be orsellinic acid (XXXV) but later (Collie 1907) amended to this formula without explanation. It might in fact be XXXVI. Some reactions of acetonedicarboxylic ester studied by Jerdan (1897, 1899) are of interest in showing that cyclizations of the C-acylation type (B) and the aldol type (A) can be made to operate in the laboratory on the same substance under slightly different conditions.

In view of the well-known ease of occurrence of the aldol condensation, and the much more difficult condensations known to occur in biological systems, such as the condensation of acetate and oxalacetate to form citrate, there is no reason to believe that reactions of this type postulated above could not readily



(XXXI)

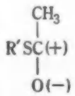
(XXXII) (XXXIII)



(XXXIV) (XXXV)

$R'CONHCH_2CH_2CONHCH_2CH_2SCOCH_3$

(XXXVII)

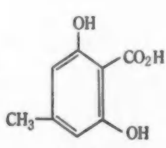


(XXXVIII)

H(+)

$(-)CH_2COSR'$

(XXXIX)



(XXXVI)

occur. The nearest analogy to the C-acylations which has been studied is the formation of acetoacetate from 2 mol. of acetate. This is known to involve the reaction of 2 mol. of acetyl coenzyme-A (partial formula XXXVII) (Lynen, Reichert, and Rueff 1951), and this substance is also the " active " acetic acid responsible for N-acetylation and for the formation of citrate from oxalacetate (Ochoa, Stern, and Schneider 1951). In the production of acetoacetate both

molecules of acetic acid require to be activated (Stadtman, Doudoroff, and Lipmann 1951), presumably in the manner usually accepted for reactions of this type in the forms XXXVIII and XXXIX. These polarizations would be assisted by the presence of the sulphur atom, which has a higher nuclear charge than oxygen and which can probably expand temporarily its outer shell of electrons. The ready separation of a proton is attested by the fact that ethylthiol acetate reacts with itself under Claisen conditions even in the presence of acetone (Baker and Reid 1929). The high acylating activity possessed by thiol acids and esters supports the supposition that polarization of the type XXXVIII occurs readily (Baddiley and Thain 1951). An " active " form of succinic acid is known (Sanadi and Littlefield 1951) and other acyl derivatives of coenzyme-A might be involved in the C-acylations postulated above, if appropriate enzymes were present.

## II. Acknowledgments

Some of this work was carried out during the tenure of the Smithson Fellowship of the Royal Society (A.J.B.). The authors are grateful to the Dunlop Rubber Co. and to the University of Sydney for a grant from the Research Fund.

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# STUDIES IN RELATION TO BIOSYNTHESIS

## II. THE STRUCTURE OF " MACROPONE "

By A. J. Birch* and Patricia Elliott*

[*Manuscript received May 28, 1953*]

*Summary*

4-*iso*Propylsalicylaldehyde (I) has been synthesized and is identical with " macropone ".

### I. Introduction

The isolation from *Eucalyptus cneorifolia* DC. oil by Reuter (1938) of a substance " macropone " having phenolic properties and carbonyl reactivity explains the red ferric colour noted by Penfold (1922). Australol (4-*iso*propyl-phenol, III), also present, does not give a colour with ferric chloride. Reuter (1950) isolated macropone in a state of purity and assigned to it the formula $C_{10}H_{12}O_2$ on the basis of analyses of numerous derivatives. It gives a Liebermann reaction, couples with diazo-compounds, and is soluble with a bright yellow colour in sodium hydroxide solution, but not in sodium carbonate. These reactions and its high volatility and intense ferric reaction support the hypothesis that it is an *o*-acylphenol. It is unlikely to be a β-diketone since it is stable to boiling 2N sulphuric acid, and it cannot be a methyl ketone since it gives no bromoform with hypobromite.

Reuter (1950) points out that the ferric reactions of *o*-acylphenols are described in the literature as purple or blue, whereas macropone gives a red with a bluish tint. However, experiments with 2-hydroxypropiophenone and salicylaldehyde show that in very dilute solution these compounds produce a red colour with a bluish tint. It will also be noted below that 5-*iso*propyl-salicylaldehyde gives a greenish brown ferric test. Reuter also points out that an aldehyde is stated to be not readily regenerated from its derivative with Girard's reagent, which he used in the purification of macropone. We find, however, that the salicylaldehyde Girard derivative is readily hydrolysed by N acid in the cold. A further objection to an aldehyde structure (Reuter 1950) was that no Schiff's test is given, but in fact salicylaldehydes do not give the normal Schiff's test (Shoesmith, Sosson, and Hetherington 1927). The molecular refraction of macropone (48·1) shows the same exaltation over the theoretical (47·3 for 3 double bonds and 1 carbonyl) as does salicylaldehyde (34·3) over the calculated (32·5), an exaltation frequently found with chelated keto-enols.

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The dark red colour of macropone 2,4-dinitrophenylhydrazone is an indication that the carbonyl is directly attached to a benzene ring. Macropone is no longer available in any quantity, since commercial exploitation of *E. cneorifolia*, found only on Kangaroo I., S. Aust., has almost ceased. Accordingly, an attempt was made to settle the constitution by synthesis on the basis of the data above and of biogenetic considerations.

Assuming macropone to be an *o*-acylphenol, two hypotheses can be put forward on biogenetic grounds. The first is that it is related to the acylphenols and β-triketones of the type of baeckeol or leptospermone or angustione. The most probable formula would be a 2-hydroxybutyrophenone or a methyl 2-hydroxypropiophenone. However, the m.p. of 2-hydroxypropiophenone 2,4-dinitrophenylhydrazone was found to be 174° C, and from a knowledge of the behaviour of homologues of such derivatives it seemed unlikely that the addition of another $CH_2$ would raise this melting point by more than 70 °C (macropone derivative m.p. 247 °C).

TABLE 1

| | Macropone | 4-*iso*Propylsalicylaldehyde |
|---|---|---|
| Boiling point .. .. .. | 131–132 °C/31 mm | 108 °C/2 mm |
| $n_D^{24}$ .. .. .. .. | 1·5480 | 1·5480 |
| Melting point (°C) | | |
| Semicarbazone .. .. | 214 | 213 |
| 2,4-Dinitrophenylhydrazone | 247 | 251 |
| Methyl ether semicarbazone | 218 | 209 |
| Phenylhydrazone .. .. | 85 | 91 |
| Ferric colour .. .. .. | Red | Red |

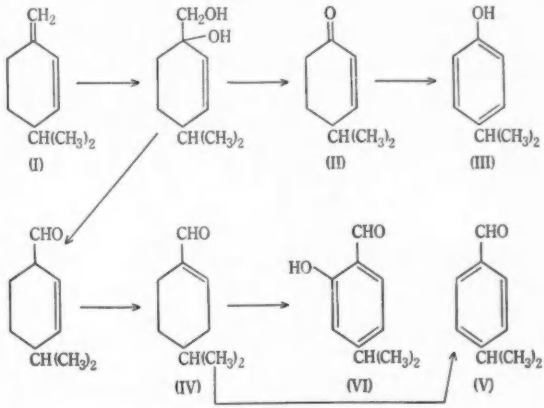
The second hypothesis is that macropone is an isoprene derivative since it occurs in an oil wholly isoprenoid in character. It would in that case probably be related to β-phellandrene, since many oxidation products of this substance are found in the oil. The most probable formula on this basis would be 4- or 5-*iso*propylsalicylaldehyde. The latter was synthesized in poor yield by a Reimer-Tiemann reaction on 4-*iso*propylphenol. The semicarbazone had m.p. 203 °C, the 2,4-dinitrophenylhydrazone m.p. 227 °C (the macropone derivatives melt at 214 and 247 °C respectively). It gave a greenish brown colour with ferric chloride. A similar synthesis of 4-*iso*propylsalicylaldehyde from 3-*iso*-propylphenol gave an oil with a cumin-like odour, closely resembling macropone in its characteristics and derivatives, as shown in Table 1.

Comparisons by mixed m.p. of the semicarbazone and 2,4-dinitrophenylhydrazone with authentic specimens confirmed their identity.

Macropone (VI) is clearly related biogenetically to β-phellandrene (I) as was expected. Many of the other oxygenated compounds in the oil may similarly be related (Berry, Macbeth, and Swanson 1937): cryptone (II), australol (III),

phellandral (IV), and cuminal (V). A number of possible sequences exist which cannot be distinguished ; the following is suggested as a probable one.

This work is a clear example of the usefulness of considering biogenetic relations in structure determination (cf. Birch and Donovan 1953), since in no other way could a choice have been made between the numerous possibilities suggested by the purely chemical evidence.



## II. Experimental

(*a*) 5-iso*Propylsalicylaldehyde*.—4-*iso*Propylphenol (2 g), chloroform (10 c.c.), and sodium hydroxide solution (20% ; 150 c.c.) were refluxed for 1 hr, the solution acidified, and steam distilled. The distillate was extracted with ether (50 c.c.) and the ether extracted with sodium hydroxide solution (10% ; 10 c.c.). Acidification gave an oil which was converted to the semicarbazone, m.p. 203 °C (160 mg). This was refluxed for 1 hr with oxalic acid solution (10%; 10 c.c.) in the presence of a little ethyl acetate. Steam distillation gave an oil which was taken up in ether, and the 5-*iso*propylsalicylaldehyde extracted with alkali. It formed a sweet smelling oil giving a greenish brown ferric test ; the *2,4-dinitrophenylhydrazone* crystallized from alcohol as bright red needles, m.p. 227 °C (Found : N, 16·2%. Calc. for $C_{16}H_{16}O_5N_4$ : N, 16·3%).

(*b*) 4-iso*Propylsalicylaldehyde*.— 3-*iso*Propylanisole (Birch and Mukherji 1949) was demethylated to 3-*iso*propylphenol by refluxing for 4 hr with hydriodic acid (*d*, 1·7). The phenol (13 g) was dissolved in potassium hydroxide solution (10% ; 200 c.c.) and refluxed with chloroform (12 c.c.) for 90 min. Worked up as above, 4-*iso*propylsalicylaldehyde semicarbazone (300 mg) was obtained, m.p. 214 °C (Found : C, 59·7 ; H, 6·7%. Calc. for $C_{11}H_{15}O_2N_3$ : C, 59·7 ; H, 6·8%). Regeneration of the aldehyde as above gave a sweet smelling oil, b.p. 108 °C/2 mm, $n_D^{24}$ 1·5480 (Found : C, 73·6 ; H, 7·6%. Calc. for $C_{10}H_{12}O_2$ : C, 73·2 ; H, 7·3%). The 2,4-dinitrophenylhydrazone formed red needles from ethanol-ethyl acetate, m.p. 251 °C (Found : N, 16·4%. Calc. for $C_{16}H_{16}O_5N_4$ : N, 16·3%). The phenylhydrazone formed cream coloured prisms, from light petroleum (b.p. 40–70 °C), m.p. 91 °C (Found : N, 11·5%. Calc. for $C_{16}H_{18}ON_2$ : N, 11·0%).

2-Methoxy-4-*iso*propylbenzaldehyde was obtained by methylation with methyl sulphate and sodium hydroxide in the usual manner. The semicarbazone crystallized from ethanol in large flat prisms, m.p. 209 °C (Found : C, 61·3 ; H, 7·1 ; N, 18·1%. Calc. for $C_{12}H_{17}O_2N_3$ : C, 61·3 ; H, 7·2 ; N, 17·9%).

(c) *Purification of Salicylaldehyde*.—Salicylaldehyde (1·0 g) was treated with the Girard-Sandulesco (1936) reagent-T in the usual manner. Regeneration from the aqueous solution was accomplished by acidification to 1N with hydrochloric acid and leaving at 20 °C for 1 hr. Extraction with ether gave salicylaldehyde (630 mg) 2,4-dinitrophenylhydrazone, m.p. 254 °C.

(d) *Isolation of Macropone*.—The crude phenolic material from *E. cneorifolia* oil (16 g) was distilled, b.p. 120–125 °C/2 mm (12 g). The distillate was heated on the steam-bath for 15 min with semicarbazide hydrochloride (3 g) and sodium acetate (3 g) in water (10 c.c.). After cooling, light petroleum (b.p. 40–70 °C ; 30 c.c.) was added and the semicarbazone which precipitated was recrystallized from ethanol. It formed colourless prisms, m.p. 214 °C, undepressed by the derivative of 4-*iso*propylsalicylaldehyde. The semicarbazone was treated with 2,4-dinitrophenylhydrazine in 2N hydrochloric acid to give macropone 2,4-dinitrophenylhydrazone, m.p. 251 °C, undepressed by the derivative of 4-*iso*propylsalicylaldehyde.

## III. Acknowledgments

The authors are grateful to Professor A. Killen Macbeth and Mr. M. J. Thompson for a gift of *E. cneorifolia* phenols.

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# STUDIES IN RELATION TO BIOSYNTHESIS

## III. THE STRUCTURE OF ELEUTHERINOL

By A. J. Birch* and F. W. Donovan*

[*Manuscript received July* 13, 1953]

*Summary*

1-Hydroxy-3-methyl-6,8-dimethoxynaphthalene (VII) has been synthesized and shown to be identical with a phenol obtained from the degradation of eleutherinol dimethyl ether. This synthesis establishes the formula VI for eleutherinol, in agreement with prediction on biogenetic grounds.

### I. Introduction

In Part I of this series (Birch and Donovan 1953) it was pointed out that an hypothesis of the biogenesis of many natural products by the head to tail linkage of acetate units could be used, like the isoprene rule, in favourable cases to indicate the most probable of alternative structures. The present work was undertaken to exemplify this statement.

The structure I (R=H) was assigned to eleutherinol, $C_{15}H_{12}O_4$, from *Eleutherine bulbosa* (Mill.) by Ebnöther, Meijer, and Schmid (1952) mainly on the results of the alkaline hydrolysis of its dimethyl ether (I, R=$CH_3$) which they interpreted as shown below. The general properties of the substance agree with the presence of a naphthochromone structure, the details of which depend on the formula assigned to the phenolic degradation product, for example, III or VII. Oxidation of the phenol with lead tetra-acetate gave in poor yield authentic IV and an *o*-quinone (V) ; all the models oxidized by the Swiss workers gave rise solely to *p*-quinones if a free *p*-position was present. Accordingly it appeared that the phenol was a 2-naphthol derivative and must be III (cf. Schmid and Burger 1952). One doubtful feature is that the acylphenol also formed in the alkaline hydrolysis must then be II although it gives the typical blue colour with 2,6-dichloroquinone chloroimide characteristic of phenols with a free *p*-position. 2-Naphthol derivatives also couple with this reagent, although the colours are usually not pure blue, but in the present case such a coupling must involve extrusion of the acetyl group. No such difficulty would be experienced with a 2-acetyl-1-naphthol derivative. Apart from these two conflicting considerations no chemical evidence is available to assist the choice between 1- and 2-naphthol types.

In the case of eleutherinol the acetate rule decides in favour of VI. The formula I can be constructed from two portions formed of acetate units in head to tail linkage, but these portions are united by a head to head linkage. Formula

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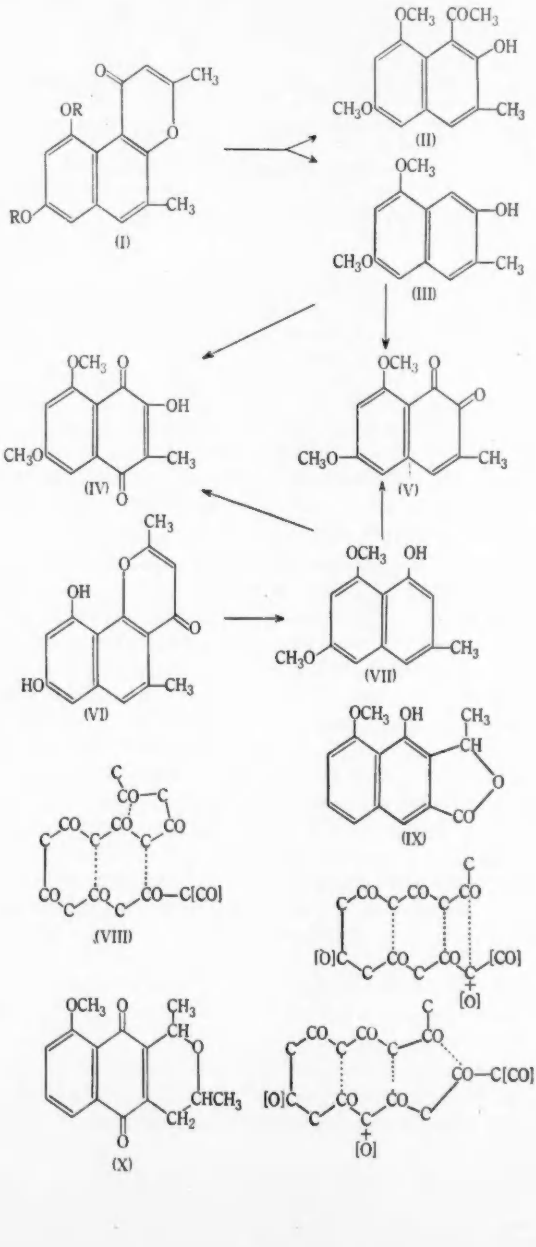
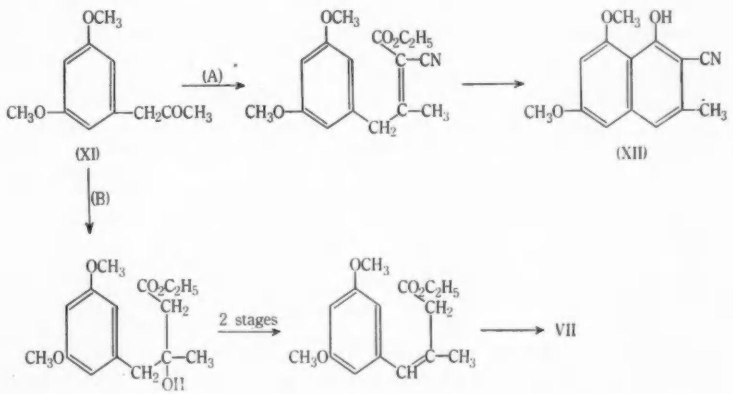

(I)
(II)
(III)
(IV)
(V)
(VI)
(VII)
(VIII)
(IX)
(X)

VI can be constructed entirely by the head to tail linkage of acetate units as in VIII. It is noteworthy also that other compounds which occur in the same plant, eleutherol (IX) and eleutherin (X), can similarly be constructed if allowance is made for the occurrence of probable oxidation-reduction reactions (cf. Birch and Donovan 1953). The same number (8) of acetate units are involved in the formation of eleutherin (X) and eleutherinol (VI). These considerations, whilst proving nothing, are weighty enough to show the desirability of synthesizing VII.

## II. Synthesis of VII

An obvious way of making 1-hydroxy-3-methyl-6,8-dimethoxynaphthalene (VII) is to build another ring on to 3,5-dimethoxyphenylacetone (XI); the usual ring-closure methods readily provide an oxygen in the correct position to form the 1-hydroxyl group. The ketone (XI) was made by modifications of standard methods: (R=3,5-dimethoxyphenyl), $RCO_2H$ (Weston and Suter 1941) ($+LiAlH_4$)$\rightarrow$$RCH_2OH$ (Adams, Harfenist, Loewe 1949) ($+SO_2Cl_2$)$\rightarrow$$RCH_2Cl$ ($+KCN$)$\rightarrow$$RCH_2CN$ (Adams, MacKenzie, Loewe 1948) ($+NaOH$)$\rightarrow$$RCH_2CO_2H$ ($+LiCH_3$)$\rightarrow$$RCH_2COCH_3$. Two methods were then tried out for the addition of the rest of the molecule:




In both cases the ring closure occurred readily with phosphoric acid-phosphorus pentoxide, but the cyanophenol (XII) formed by route (A) was very resistant to hydrolysis and the cyano-group could not be removed. In the case (B) the pure intermediates were not isolated, but the final product (Table 1) analysed correctly and had the same melting point and ultraviolet absorption spectrum as the eleutherinol degradation product (Ebnöther, Miejer, and Schmid 1952)* (Table 1).

* See footnote to Table 1.

TABLE 1

| 1-Hydroxy-3-methyl-6,8-dimethoxynaphthalene (VIII) M.p. 82 °C | | Phenol from Eleutherinol* M.p. 82–84 °C | |
|---|---|---|---|
| $\lambda_{max.}$ (m$\mu$) | log $\varepsilon_{max.}$ | $\lambda_{max.}$ (m$\mu$) | log $\varepsilon_{max.}$ |
| 234 | 4·70 | 234 | 4·68 |
| 298 | 3·73 | 300 | 3·70 |
| 320 | 3·59 | 320 | 3·55 |
| 335 | 3·63 | 333 | 3·60 |
| 258 | 3·26 | 258 | 3·18 |
| 315 | 3·54 | 314 | 3·51 |
| 327 | 3·49 | 328 | 3·46 |

* The values are approximate since they are taken from small-scale curves.

The phenol (VII) was not extractable from ether with sodium hydroxide solution and gave a pure blue colour with 2,6-dichloroquinone chloroimide, properties also reported for the degradation phenol.

TABLE 2

| 2-Hydroxy-3-methyl-6,8-dimethoxy-1,4-naphthaquinone (IV) M.p. 203–204 °C | | *p*-Quinone from Eleutherinol* M.p. 205–206 °C | |
|---|---|---|---|
| $\lambda_{max.}$ (m$\mu$) | log $\varepsilon_{max.}$ | $\lambda_{max.}$ (m$\mu$) | log $\varepsilon_{max.}$ |
| 266 | 4·06 | 265 | 4·20 |
| 300 | 4·04 | 300 | 4·20 |
| 368 | 3·57 | 365 | 3·66 |
| **3-Methyl-6,8-dimethoxy-1,2-naphthaquinone (V) M.p. 198–199 °C** | | ***o*-Quinone from Eleutherinol* M.p. 199–201 °C** | |
| $\lambda_{max.}$ (m$\mu$) | log $\varepsilon_{max.}$ | $\lambda_{max.}$ (m$\mu$) | log $\varepsilon_{max.}$ |
| 266 | 4·17 | 264 | 4·16 |
| 408 | 3·95 | 408 | 3·90 |

* The values are approximate since they are taken from small-scale curves.

Oxidation of the phenol with lead tetra-acetate under the conditions described by Ebnöther, Meijer, and Schmid (1952) gave a mixture of the *p*-quinone (IV) and the *o*-quinone (V) also having the correct melting point and absorption spectra (Table 2).

None of the authentic material is available for comparison with VII but there seems little doubt that eleutherinol is correctly represented by VI, especially as the only evidence in support of I (R = H) is destroyed by the discovery that authentic VII gives rise to some *o*-quinone with lead tetra-acetate.

## III. Experimental

Melting points are uncorrected.

(*a*) *3,5-Dimethoxyphenylacetic Acid.*—3,5-Dimethoxyphenylacetonitrile (5·7 g) and 5N hydrochloric acid were refluxed for 7 hr. After cooling the product was taken up by several ether extractions, and then removed from the ether by sodium carbonate solution. Acidification gave 3,5-dimethoxyphenylacetic acid (98%; 5·85 g). Crystallization from water gave white needles, m.p. 99–100 °C; Mauthner (1925) gives m.p. 99–100 °C.

(*b*) *3,5-Dimethoxyphenylacetone.*—A solution of the dimethoxyphenylacetic acid (2·2 g) in dry ether (60 c.c.) was rapidly added to a solution of methyllithium from lithium (0·75 g) and methyl iodide (11·4 g) in ether (60 c.c.) and refluxing continued for 10 min. The ether was then washed with water and sodium carbonate, and on evaporation an oil (0·85 g) was left which was purified in the usual way through the bisulphite compound to give 3,5-dimethoxyphenylacetone; semicarbazone, m.p. 134 °C; Adams, Harfenist, and Loewe (1949) give m.p. 134·5–135·3 °C. Acidification of the water and carbonate washings gave dimethoxyphenylacetic acid (1·2 g). The yield of ketone varied considerably and was as high as 76%, not taking into account recovered acid.

(*c*) *2-Cyano-1-hydroxy-3-methyl-6,8-dimethoxynaphthalene.*—3,5-Dimethoxyphenylacetone (3·1 g), ethyl cyanoacetate (2·0 g), ammonium acetate (0·60 g), and acetic acid (1·30 c.c.) in benzene (25 c.c.) was refluxed with azeotropic distillation for 3 hr. The benzene was removed under reduced pressure, the oil taken up in ether, washed with dilute sodium hydroxide and water, dried, and the ether removed. The ethyl 2-cyano-4(3′,5′-dimethoxyphenyl)methylbut-2-enoate (3·64 g) was not purified but used directly for the next stage.

A mixture of the ester (1·0 g) and a solution of phosphorus pentoxide (5·0 g) in syrupy phosphoric acid (5 c.c.) were kept at 85–90 °C for 4·5 min with stirring, cooled, diluted with water (200 c.c.), and the gummy solid (0·62 g) removed by filtration. It was sublimed at 150–170 °C/1·0 mm and crystallized from ethanol, m.p. 185–186 °C (Found: N, 5·9%. Calc. for $C_{14}H_{13}O_3N$: N, 5·8%). Attempts to obtain a uniform product either by acid or alkaline hydrolysis were unsuccessful.

(*d*) *1-Hydroxy-3-methyl-6,8-dimethoxynaphthalene.*—Zinc needles (0·9 g) were added to ethylbromoacetate (1·95 g) and 3,5-dimethoxyphenylacetone (1·8 g) in dry benzene (50 c.c.), and the mixture refluxed for 2 hr. The mixture was then treated with dilute sulphuric acid, the organic layer taken up in ether, well washed with water, and the solvent removed. The product, presumably ethyl 3-hydroxy-3-methyl-4(3′,5′-dimethoxyphenyl)butyrate was an oil (2·38 g) no longer showing ketonic reactivity. It could not be crystallized. Dehydration was effected by refluxing in benzene (50 c.c.) with phosphorus pentoxide (1·5 g), and the product purified by passage through a column of alumina to give a viscous oil (1·65 g). Attempts to hydrogenate it with Pd—C catalyst resulted in the uptake of only about 30% of the theoretical amount of hydrogen. The product was hydrolysed with sodium hydroxide (10%; 15 c.c.) and the resulting acid (1·08 g) kept at 100 °C for 5 min and at 125 °C for a further 5 min with a solution of phosphorus pentoxide (4·0 g) and phosphoric acid (4 c.c.). Worked up as usual, the ethereal solution was washed with sodium hydroxide (in which the expected phenol is not soluble) and the ether removed to leave a crystalline product. The 1-hydroxy-3-methyl-6,8-dimethoxynaphthalene was chromatographed on alumina in light petroleum (b.p. 40–70 °C) (0·70 g) (Found: C, 71·6;

H, 6·5%. Calc. for $C_{13}H_{14}O_3$: C, 71·5; H, 6·5%). It was insoluble in sodium hydroxide solution and an acetone solution gave an immediate deep blue colour with 2,6-dichloroquinone chloroimide in a buffer at pH 9. The ultraviolet absorption is recorded above.

Oxidation with lead tetra-acetate was carried out as described by Ebnöther, Meijer, and Schmid (1952), except that the alkali-insoluble *o*-quinone was not distilled but was washed first with a little ether and then recrystallized from acetone. The properties of the oxidation products are described above.

## IV. Acknowledgments

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# THE STRUCTURE OF AROMADENDRENE. I

By A. J. Birch* and F. N. Lahey†

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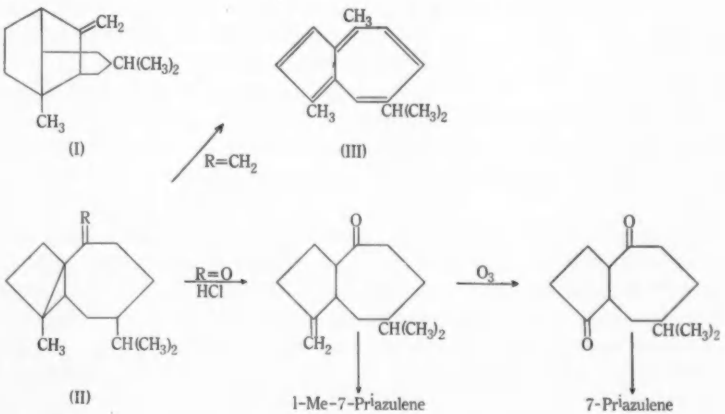
*Summary*

The reactions of aromadendrene do not agree with either formulae I or II ($R = CH_2$). On the basis of the reactions of *apo*aromadendrone, assisted by the use of infra-red spectra the formula IV is tentatively proposed. A new stereoisomeric form of *apo*-aromadendrone has been obtained.

## I. Introduction

The sesquiterpene hydrocarbon aromadendrene, $C_{15}H_{24}$, gives rise on dehydrogenation to guaiazulene(1,4-dimethyl-7-*iso*propylazulene, III), and on ozonolysis to formaldehyde and a ketone " aromadendrone ", $C_{14}H_{22}O$, more correctly styled *apo*aromadendrone. The general properties of the hydrocarbon are in agreement with a tricyclic structure bearing a $=CH_2$ group. Treibs and




Barchet (1950) removed successively the $=CH_2$ and methyl group, and dehydrogenated the substances so obtained. On the basis of absorption spectra they formulated the resulting azulenes as the 1-methyl-7-*iso*propyl- and the 7-*iso*propyl- respectively. They considered II to be the most probable constitution, and formulated the reactions as shown below. Their conclusions invalidated

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the formulae of Radcliffe and Short (1938) and Harper (1947) which both equate the $=CH_2$ to the 1-Me of guaiazulene (III).

The formula II (R $=$O) for *apo*aromadendrone does not, however, account for its stability and ultraviolet absorption spectrum. It is, for example, unaffected by alcoholic sulphuric acid under conditions more drastic than those used by Baeyer (1896) to cause fission of the *cyclo*propane ring in carone, which is similarly situated adjacent to a carbonyl group. The ultraviolet absorption spectrum of *apo*aromadendrone has an $\varepsilon$ of 25 at 225 m$\mu$, and of $\alpha$-*apo*aromadendrone (see below) of 95 at 212 m$\mu$. This does not indicate the conjugation of the *cyclo*propane ring with the carbonyl group, since Klotz (1944) showed that in such cases there is considerable absorption below 225 m$\mu$: carone and i-cholestenone have $\varepsilon$ values of 195 at 225 m$\mu$, and of about 1000 at 216 m$\mu$.

The presence of $-CH_2-$ adjacent to the carbonyl group of *apo*aromadendrone was apparently indicated by the production of a benzylidene derivative (Radcliffe and Short 1938) but the catalyst used was hydrogen chloride, shown by Treibs and Barchet (1950) to cause ring fission. On the basis of this evidence, and assuming the benzylidene derivative to come from *isoapo*aromadendrone, Birch (1950) proposed the formula I. However, additional evidence now obtained disproves both this and formula II.

Re-examination of the ozonolysis of aromadendrene led to the surprising conclusion that *apo*aromadendrone, which has been used for many years for identifying the sesquiterpene, is not the initial product. Careful work gave a new ketone $\alpha$-*apo*aromadendrone which is a stereoisomer of *apo*aromadendrone, into which it is readily converted by alkali or by heat. The properties are compared below. The melting point of the ketones, and their derivatives are depressed by admixture. *apo*Aromadendrone, m.p. 83–84 °C, $[\alpha]_D$ $+3\cdot5°$, oxime, m.p. 103 °C; $\alpha$-*apo*aromadendrone, m.p. 71–72 °C, $[\alpha]_D$ $-5\cdot6°$, oxime, m.p. 138 °C.

This facile isomerization is evidence either that an asymmetric centre adjacent to the carbonyl has been inverted to a more stable configuration, or that a double bond has moved into or away from the vicinity of the carbonyl group. Neither of the ketones shows any ultraviolet absorption indicative of an $\alpha\beta$-unsaturated ketone, but merely the low intensity carbonyl band at $\lambda_{max.}$ 285 m$\mu$ (cf. also Naves and Perrottet 1940). Formula II has no invertible centre adjacent to the carbonyl unless the ring is broken and an $\alpha\beta$-unsaturated ketone might be expected in that event.

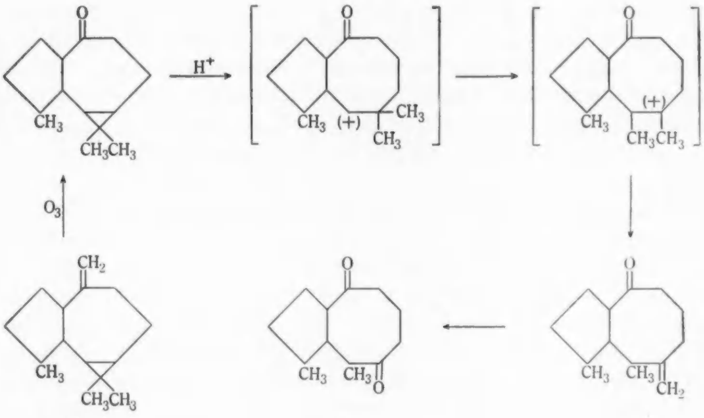
Evidence that the centre involves the expected $-CH<$ was obtained by the action of amyl nitrite and sodium ethoxide. There resulted in good yield, after alkaline hydrolysis, an oximino-acid, $C_{14}H_{23}O_3N$, from either ketone. This acid has no double bond conjugated with the oxime group since it possesses no absorption maximum between 210–350 m$\mu$ (cf. Evans and Gillam 1943) and the 2,4-dinitrophenylhydrazone to which it gives rise on vigorous treatment with 2,4-dinitrophenylhydrazine in 2N hydrochloric acid has a maximum at 367 m$\mu$.

The derivative of a saturated ketone has $\lambda_{max.}$ *c.* 363 mμ and of an αβ-unsaturated ketone $\lambda_{max.}$ *c.* 380 mμ (Braude and Jones 1945). Again there is no evidence of ring fission. Hydrolysis of the oxi .e gives an oily keto-acid from which the oxime can be regenerated. The ca oonyl band in the infra-red is at 5·75μ indicative of the presence of the gro up in a *cyclo*pentane ring. This conclusion is in accord with the situation of the $=CH_2$ of aromadendrene in the 4-position of the azulene skeleton, adjacent to a $-CH<$ in the ring junction, provided the asymmetric centre is not in the 5-position. It was therefore necessary to re-examine the question of the presence of a $-CH_2-$ there. This has now been done by condensing *apo*aromadendrone and α-*apo*aromadendrone with ethyl formate to produce the same hydroxymethylene derivative converted by aniline to the anilinomethylene derivative m.p. 141–142 °C. This was hydrolysed to regenerate *apo*aromadendrone, so that no alteration could have taken place in the molecular skeleton during the condensation. Formula I is therefore excluded.

The observation of Treibs and Barchet (1950) that the action of dry hydrogen chloride on *apo*aromadendrone leads to fission of a ring can only be readily explained if a *cyclo*propane ring is present, although, as shown above, this is unlikely to be conjugated with the carbonyl group. Ozonolysis of the resulting *isoapo*aromadendrone produces a diketone, $C_{13}H_{20}O_2$, m.p. 98 °C with loss of one carbon atom; the substance must therefore contain a new $=CH_2$. This has been confirmed by the presence of infra-red bands at 5·65, 6·08, and 11·27μ. Repetition of these experiments has led also to the isolation of an isomeric diketone, $C_{13}H_{20}O_2$, m.p. 125 °C, which does not appear to be a stereoisomer of that melting at 98 °C. The results would appear to indicate that one end of the bond which has been broken is attached to a carbon bearing a methyl group, that is, to the 1-position as postulated by Treibs and Barchet (loc. cit.), or to the *iso*propyl group.

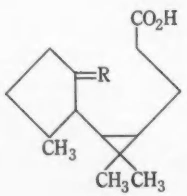
A careful examination of the carbonyl region in the infra-red spectrum of a solution of the diketone, m.p. 125 °C in chloroform gave puzzling results. Only *one* sharp band was observed at 5·909μ (chloroform solution) corresponding to only one type of carbonyl group, probably situated in a large ring. Šorm, Dolejš, and Plíva (1950) record the value of 5·907μ for a carbonyl group in the *cyclo*heptane ring of cis-[0,3,5]-*bicyclo*decanone and 5·910μ for a carbonyl group in *cyclo*-octanone (chloroform solution). A carbonyl group in the 1-position should produce a band at about 5·77μ (*cyclo*pentanone ring) and in an acetyl group a band at about 5·85μ. The former should be present on the formulation of Treibs and Barchet, and the latter would result from the presence of an *iso*propenyl group in *isoapo*aromadendrone. We must therefore conclude that both carbonyl groups are situated in the same *cyclo*heptandione or *cyclo*-octandione ring. An explanation based on a formula such as IV for aromadendrene is formulated below: The formulae are arbitrarily chosen; aromadendrene might have the $-CMe_2-$ attached at 6,7- instead of 7,8-, and the direction of fission of the *cyclo*propane ring is unknown.

This formulation would require the production of azulenes by the dehydrogenation of V and VI (cf. Treibs and Barchet 1950), for which there is at present no known analogy. Such a ring contraction is not improbable but the azulenes produced would not necessarily have the formulae assigned to them by Treibs and Barchet.* In particular they would carry a 1-Me group, the presence of which would also seem to be in accord with the absorption spectra recorded by these authors.

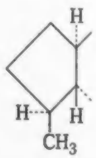


(IV) (V) (VI)

The ready conversion of α-*apo*aromadendrone to *apo*aromadendrone shows that the junction of the *cyclo*pentane and *cyclo*heptane rings in aromadendrene must be in the unstable *trans*-configuration. Regeneration of the oximino-acid (VII, R=NOH) from the keto-acid (VII, R=O) after treatment with alkali indicates that this is in the sterically stable form with the 1-Me trans to the other group attached to the *cyclo*pentane ring. The steric configuration of this part of the molecule is then probably that shown in VIII, or its mirror image.



(VII)



(VIII)

* The diketone, m.p. 98 °C, may still have an azulene skeleton, thus explaining these results; urther examination of the question is in progress.

## II. EXPERIMENTAL*

(*a*) α-apo*Aromadendrone*.—Aromadendrene (5 g), b.p. 122 °C/10 mm, $n_D^{25}$ 1·4953, $d_4^{25}$ 0·9111, $[\alpha]_D$ +7·54°, in pure ethyl acetate (25 c.c.) was cooled in ice and 5% ozonized oxygen passed until ozone emerged freely. To the solution was added acetic acid (10 c.c.), water (2 c.c.), and zinc dust (5 g) with vigorous stirring. An exothermic reaction commenced after several minutes, and the temperature was kept below 30 °C by cooling. After 30 min the solution was decanted from the zinc, taken up in ether (50 c.c.), and washed first with water and then with sodium hydrogen carbonate solution until free of acetic acid. It was then dried with sodium sulphate and evaporated under reduced pressure. The residue could not readily be induced to crystallize until seed crystals became available. It was warmed on the steam-bath for 15 min with a solution of sodium acetate (3 g), semicarbazide hydrochloride (3 g), water (5 c.c.), and methanol (10 c.c.). After addition of water (10 c.c.) and leaving in the refrigerator the solid mass was removed by filtration, washed with cold methanol, and recrystallized from methanol as colourless needles, m.p. 190–191 °C (2·8 g). The semicarbazone was decomposed by shaking in the cold with ether (20 c.c.) and hydrochloric acid (0·5N ; 20 c.c.) until the solid had all passed into solution (about 3 hr). The ether solution was washed with sodium hydrogen carbonate, dried, and then evaporated under reduced pressure. The residue crystallized and was recrystallized twice from aqueous methanol as colourless plates, m.p. 71–72 °C, $\lambda_{max.}$ 285 mμ, $\varepsilon_{max.}$ 22·3, $[\alpha]_D$ —5·6° (in ethanol) (Found : C, 81·2 ; H, 11·0%. Calc. for $C_{14}H_{22}O$ : C, 81·5 ; H, 10·7%). The oxime, prepared by heating with hydroxylamine acetate in methanol from the ketone or the crude starting material formed colourless needles from aqueous methanol, m.p. 138 °C (Found : C, 76·0 ; H, 10·6%. Calc. for $C_{14}H_{23}ON$ : C, 76·0 ; H, 10·4%). By heating with a solution of sodium (0·5 g) in ethanol (10 c.c.) on the steam-bath for 10 min the α-*apo*aromadendrone was converted to *apo*aromadendrone, m.p. 82–83 °C, $[\alpha]_D$ +3·5° (in acetone) oxime, m.p. 103 °C, both of which substances produced marked depression in the m.p. of α-*apo*aromadendrone and its derivative. The ketones had similar odours, but the α-compound is more like camphor and less musty.

(*b*) *Anilinomethylene*apo*aromadendrone*.—α-*apo*Aromadendrone (or *apo*aromadendrone) (4 g) was added together with ethyl formate (8 c.c.) to dry sodium methoxide (from the metal, 0·7 g, dried at 180 °C/30 min) and ether (20 c.c.). The mixture was left overnight and poured on to crushed ice. The aqueous solution was separated, the ether layer washed with ice-cold sodium hydroxide solution (3%) and the combined aqueous solutions acidified and the product taken up in ether. Evaporation of the solvent left hydroxymethylene*apo*aromadendrone as a pale yellow oil (3·5 g) $[\alpha]_D$ +44° which eventually crystallized, m.p. 29–31 °C (Found : C, 76·6 ; H, 9·6%. Calc. for $C_{15}H_{22}O_2$ : C, 76·9 ; H, 9·4%). It gave a bright wine-red colour with ferric chloride. A few drops mixed with aniline gave *anilinomethylene*apo*aromadendrone* as pale yellow needles, crystallized from methanol, m.p. 141–142 °C (Found : C, 81·65 ; H, 8·7 ; N, 4·8%. Calc. for $C_{21}H_{27}ON$ : C, 81·55 ; H, 8·7 ; N, 4·5%). Hydrolysis first with boiling 2N hydrochloric acid (30 min) and then with N sodium hydroxide (1 hr) regenerated *apo*aromadendrone, m.p. 84–85 °C $[\alpha]_D$ +3·5°.

(*c*) *Oximino-acid* (*VII, R=NOH*).—*apo*Aromadendrone (1·6 g) was added to a solution of sodium (0·29 g) in ethanol (20 c.c.) and, after cooling in ice, amyl nitrite (1·1 g) was added in three successive portions. The mixture was left 1 hr in ice and 1 hr at room temperature and then poured into boiling water (100 c.c.) and boiling continued for 15 min. The solution was extracted with ether and the aqueous layer acidified and extracted with ether to yield an *oximino-acid* (1·4 g), m.p. 167–168 °C (decomp.) from methanol, $[\alpha]_D$ +12° (Found : C, 66·35 ; H, 9·0 ; N, 5·6%. Calc. for $C_{14}H_{22}O_3N$ : C, 66·4 ; H, 9·1 ; N, 5·5%). After heating with 2,4-dinitrophenylhydrazine in 2N hydrochloric acid on the steam-bath for 1 hr a *2,4-dinitrophenylhydrazono-acid* was obtained and recrystallized from methanol as orange needles, m.p. 208 °C (Found : N, 14·8%. Calc. for $C_{20}H_{26}O_4N_4$ : N, 14·7%). The compound had $\lambda_{max.}$ 367 mμ ; $\varepsilon_{max.}$

* Some of the preliminary experimental work was carried out by Miss E. Klein (Mrs. Nelson) (Ph.D. Thesis, University of Melbourne (1951).)

23,000 ; $\lambda_{min.}$ 298 m$\mu$, $\varepsilon_{min.}$ 1540 (in chloroform). After heating with 1N hydrochloric acid for 30 min an oily keto-acid was obtained (Found : C 70·3 ; H, 9·4%. Calc. for $C_{14}H_{22}O_3$ : C, 70·6 ; H, 9·2%) which gave rise to the above oximino-acid, m.p. 167–168 °C, $[\alpha]_D$ +12° on treatment with hydroxylamine acetate and to the 2,4-dinitrophenylhydrazono-acid with Brady's reagent. After refluxing the keto-acid with sodium hydroxide solution (5%) for 30 min the crystalline oximino-acid $[\alpha]_D$ +12° could be regenerated.

(*d*) isoapo*Aromadendrone*.—*apo*Aromadendrone (5 g) was moistened with light petroleum (b.p. 40–60 °C) and hydrogen chloride passed into the mixture with ice-cooling until 5% increase in weight had occurred. It was then left in the refrigerator, first becoming completely liquid and then resolidifying in about 24 hr. Sodium carbonate solution (1N ; 50 c.c.) was added and the mixture heated on the steam-bath for 30 min. Ether extraction gave an oil which was passed through alumina (40 g) in light petroleum (b.p. 40–60 °C). After evaporation of the light petroleum the residue solidified and was recrystallized in the refrigerator from a little light petroleum (b.p. 40–60 °C) as long flat prisms, m.p. 55–58 °C (4·1 g). Further crystallization raised the m.p. to 61–62 °C (Found : C, 81·3 ; H, 10·6%. Calc. for $C_{14}H_{22}O$ : C, 81·5 ; H, 10·7%).

(*e*) *Ozonolysis of* isoapo*Aromadendrone*.—Ozonolysis as described by Treibs and Barchet (1950) gave rise to the diketone, m.p. 98 °C (Found : C, 75·4 ; H, 10·2%. Calc. for $C_{13}H_{20}O_2$ : C, 75·0 ; H, 9·9%). In one case, crude *isoapo*aromadendrone, m.p. 55–58 °C, was employed, and on chromatography of the product on alumina in light petroleum (b.p. 40–60 °C) a second *ketone* was obtained from the final eluates. It crystallized from light petroleum (b.p. 40–60 °C) : ethyl acetate (2 : 1) in massive prisms, m.p. 125 °C (Found : C, 75·3 ; H, 10·2%. Calc. for $C_{13}H_{20}O_2$ : C, 75·0 ; H, 9·9%). The infra-red spectrum had a band at 5·90 $\mu$ (" Nujol " mull) or 5·909 $\mu$ (chloroform solution).

## III. Acknowledgments

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# NATURAL DERIVATIVES OF FURAN

## I. NGAIONE

By A. J. Birch,* R. Massy-Westropp,* and S. E. Wright†

[*Manuscript received May 28, 1953*]

*Summary*

Ngaione has been isolated from the volatile oil of *Myoporum acuminatum* R.Br. and assigned the formula II. Different specimens of the oil contained other ketones.

### I. The Structure of Ngaione

Several species of the genus *Myoporum*, endemic to Australia and New Zealand, are toxic to stock and apparently owe at least part of their toxicity to the presence of furan ketones. *Myoporum laetum* Forst. f. ("ngaio") yields a volatile oil which is toxic (Cunningham and Hopkirk 1945), and so does *M. acuminatum* R.Br. (Riek and Wright 1946). The active constituent of the former is the sesquiterpene ketone, ngaione, and in the latter it forms part of the higher-boiling sesquiterpenoid fraction. An examination has now been made of this fraction.

A specimen of *M. acuminatum* was obtained from the Goondiwindi district, Queensland, with the generous assistance of the C.S.I.R.O. Phytochemical Survey, and steam distilled by Mr. M. D. Sutherland. The volatile oil (179 g) on careful distillation gave a series of fractions (total about 40 g) which appeared to consist of one pure substance. The properties were similar to those recorded for ngaione (McDowall 1925, 1927, 1928; Brandt and Ross 1949), and it was identified as this ketone by comparison of the *p*-nitrophenylhydrazone, m.p. 100 °C, with an authentic specimen, m.p. 102 °C, supplied by Mr. M. D. Sutherland.

| Source | B.p. | $n_D^{15\cdot5}$ | $d_{15\cdot5}^{15\cdot5}$ | $[\alpha]_D$ |
|---|---|---|---|---|
| *M. laetum* (ngaione) .. | .. 181–183 °C/27 mm | 1·4804 | 1·0276 | —26·2° |
| *M. acuminatum* .. | .. 130 °C/1 mm | 1·4832 | 1·027 | —33·9° |

Ngaione was extensively investigated by these workers, but no complete formula has been put forward; Brandt and Ross (1949) suggested the partial formula I. The two largest oxidation fragments from ngaione, $C_{15}H_{22}O_3$, are 3-furoic acid (IV) and *iso*valeric acid (V), which both have isoprene skeletons. It is likely therefore, that the compound is isoprenoid and has a sesquiterpene skeleton; a number of natural furans are known to have a monoterpene skeleton (perillene, elsholtzione, clausenan, and menthofuran). McDowall (1925, 1927,

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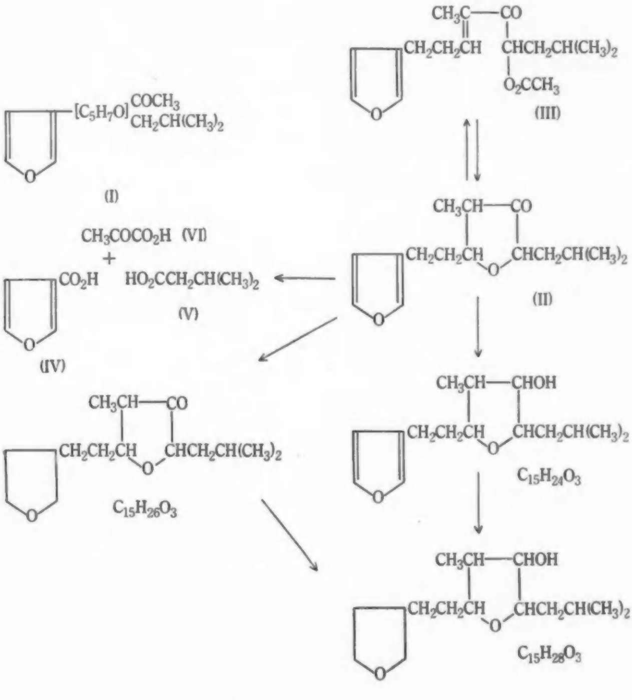
1928) established that it is a monoketone, and the molecular refraction $[R_L]_D$ 69·1 is in accord with the presence of two double bonds, one carbonyl, and two ether oxygens (calculated 69·4). Reduction with sodium produces a secondary mono-alcohol, $C_{15}H_{24}O_3$, (ngaiol) which can be catalytically hydrogenated to tetrahydrongaiol, $C_{15}H_{28}O_3$. Hydrogenation of ngaione gives the ketone tetrahydrongaione, $C_{15}H_{26}O_3$, reducible by sodium to tetrahydrongaiol. Ketonic reactivity in this series is very low and the action of sodium hypobromite on tetrahydrongaione gives a compound $C_{15}H_{24}O_3Br_2$, so that the presence of an acetyl group is unlikely. Ngaione slowly reduces Fehling's solution or ammoniacal silver nitrate, which may indicate the presence of a potential α-ketol structure. McDowall investigated a number of other reactions, but rather indefinite products do not permit of further conclusions being drawn. He considered ngaione to be a ketone containing two oxide rings and two double bonds, probably in a furan ring.

Brandt and Ross (1949) confirmed the presence of a furan ring by the isolation of 3-furoic acid (IV) after oxidation with potassium ferricyanide; they also obtained *iso*valeric acid (V) and pyruvic acid (VI). It is unlikely that pyruvic acid would be formed from *iso*valeric acid, and three methyl groups are probably present in ngaione. A highly significant reaction is the action of acetic anhydride and sodium acetate to form an acetate "*iso*ngaione acetate", $C_{15}H_{21}O_2(OCOCH_3)$, which gives rise again to ngaione or an isomer on alkaline hydrolysis (Brandt and Ross 1949). We assume that an oxide ring is opened to give the acetate of an unsaturated ketol (III) ring closure occurring again by a Michael-type addition onto the αβ-double bond as soon as the hydroxyl is regenerated. This supposition has now been confirmed by examining the ultraviolet absorption of the acetate. Ngaione itself shows no selective absorption in the region 230–300 mμ, but *iso*ngaione acetate has a peak at $\lambda_{max.}$ 238 mμ (ε 8100), which is in accord with the presence of an αβ-unsaturated ketone group and is in the calculated position ($\lambda_{max.}$ 237 mμ) for α-, β-substituents on the double bond as in III (Woodward 1941). On the assumption of a normal sesquiterpene skeleton the only probable formula is II. A theoretical but unlikely possibility has the $CH_3CH$ and C=O groups interchanged; the skeleton would then be incompletely isoprenoid. The formulae below illustrate the reactions discussed.

## II. Variability of *Myoporum* Oils

Two other specimens of *M. acuminatum* which were examined gave oils containing at most traces of ngaione. Sample (A), collected in 1945 at Mundubbera, Queensland, gave a fraction having the following characteristics: b.p. 112–115 °C/1 mm, $n_D^{15\cdot5}$ 1·514, $d_{15\cdot5}^{15\cdot5}$ 1·109, $[\alpha]_D$ −100°, $\lambda_{max.}$ 230 mμ, $\varepsilon_{max.}$ 7692 (calc. on mol. wt. 246). The ultraviolet absorption corresponds to the presence of an αβ-unsaturated ketone grouping with one substituent on the double bond (Woodward 1941), and the substance shows ketonic reactivity although no crystalline derivatives could be obtained. Analyses indicate a molecular formula $C_{16}H_{22}O_2$, but the formula might be $C_{15}H_{20}O_2$: McDowall

(1925) originally assigned a $C_{16}$-formula to ngaione before crystalline derivatives were obtained. Hydrogenation gives rise to a hexahydro-derivative still showing ketonic reactivity. Oxidation with alkaline ferricyanide produces a low yield of 3-furoic acid and *iso*butyric acid, while permanganate in acetone forms *iso*valeric acid and oxalic acid together with an unidentified neutral solid,




m.p. 171 °C which may be $C_{13}H_{18}O_4$. The infra-red absorption of this compound indicates the presence of hydroxyl, saturated carbonyl, and *gem*-dimethyl groups. It is impossible at present to put forward with any certainty a formula for the original ketone, or even to be certain that it consists essentially of one substance.

Another specimen of *M. acuminatum* (B) collected in August 1951, 12 miles south of Chinchilla, Queensland, was found to contain a complex mixture of ketones again lacking in ngaione. Small quantities of 3-furoic acid separated in the column during distillation, so furan derivatives must be present. From Table 3 (Section III) it can be seen that possibly four or five compounds are present, some of which cannot contain a furan ring. The position of the absorption maxima at 237 mμ indicates the αβ-unsaturated nature of the ketone group, two substituents being present on the double bond.

The reason for the variation in the oils is unknown, but is under investigation, and further work is planned on the oil constituents when supplies become available. The toxicity of the oils may be due to the $\alpha\beta$-unsaturated ketone grouping (or a potential one as in ngaione) rather than to the furan ring as such.

## III. Experimental

(*a*) *Isolation of Ngaione.*—Fresh leaves of *M. acuminatum* (150 lb), collected December 1952 in the Goondiwindi district, Queensland, were distilled in steam for 20 hr. The oil (*c.* 180 g) was washed with sodium carbonate solution, and the extract gave a small amount of 3-furoic acid, m.p. 120 °C. After washing with sodium hydroxide to remove a small amount of phenolic material, the oil was dried and fractionated through a column (55 by 1·5 cm) filled with stainless steel gauze Dixon rings and fitted with a constant reflux head and variable take-off. The results of the distillation are shown in Table 1 (pressure 0·5–1 mm).

Table 1

| Fraction | Mass (g) | Still-Head (°C) | $n_D^{20}$ | $[\alpha]_D$ (deg.) | $d_{15}^{15}$ |
|---|---|---|---|---|---|
| I | 7·4 | 72 | 1·4882 | — 5·7 | |
| II | 7·9 | 82–84 | 1·4918 | | |
| III | 8·1 | 84 | 1·4982 | —14·7 | |
| IV | 8·6 | 86 | 1·5036 | | |
| V | 8·4 | 86 | 1·5061 | —16·6 | |
| VI | 7 | 90 | 1·5061 | | |
| VII | 7·8 | 90 | 1·5061 | — 0·1 | |
| VIII | 6·3 | 90 | 1·5051 | | |
| IX | 6·9 | 98–100 | 1·5013 | | 0·9685 |
| X | 9·5 | 100 | 1·5013 | —12·4 | |
| XI | 13·9 | 104 | 1·5000 | | |
| XII | 12·9 | 112–114 | 1·4849 | —34·6 | |
| XIII | 10·3 | 120 | 1·4824 | | |
| XIV | 11·0 | 122 | 1·4824 | —32·2 | |
| XV | 9·8 | 122 | 1·4849 | | |
| XVI | 11·7 | 130 | 1·5002 | —14·4 | |
| XVII | 11·9 | 140 | 1·4848 | | |
| XVIII | — | 150 | | | |

Fraction XVII partially crystallized on refrigeration, and an examination of this material will be reported later. Fractions IX–XIV were refractionated through another column, designed according to Shorland (1952), (Table 2) (pressure 0·5–1 mm).

From the graph of refractive indices and rotations it was concluded that fractions IX–XIV (Table 2) are essentially identical, and further work was carried out on this material. The ultraviolet absorption curve showed no peak between 230–320 m$\mu$ (cf. Brandt and Ross 1949).

The substance (2·35 g) was treated in the standard manner with the reagent P of Girard and Sandulesco (1936) (3·7 g) for 90 min. Only about 15% of ketonic fraction was obtained, but on two repetitions of the extraction procedure about the same proportion was extracted, so it appears that the low yield is due to a hindered carbonyl group. The extracted material gave rise to a pale yellow *p*-nitrophenylhydrazone, m.p. 100 °C after passage of a solution in ethyl acetate through a short column of alumina. This m.p. was undepressed by an authentic specimen, m.p.102 °C kindly supplied by Mr. M. D. Sutherland.

A sample of leaves submitted to preliminary air drying on distillation gave a higher proportion of high-boiling material, and the refractive indices of the fractions were consistently higher. The oil did not, however, resemble those from samples (A) or (B) below.

(*b*) iso*Ngaione Acetate*.—This was prepared according to Brandt and Ross (1949): b.p. 142–144 °C/0·5 mm, $n_D^{20}$ 1·4866, $\lambda_{max.}$ 236–238 mμ, $\varepsilon_{max.}$ 8100. On hydrogenation, using palladium charcoal, there was a distinct slowing in the rate after 1 mol of hydrogen had been absorbed, and the peak at 237 mμ had disappeared in the product.

(*c*) *Specimen* (*A*).—Dried *M. acuminatum* leaves from Mundubbera, Queensland, were treated as above, and a middle fraction of the oil was selected for investigation, since it could not be further separated in the column. It had the constants: b.p. 112–115 °C/1 mm, $d_{15 \cdot 5}^{15 \cdot 5}$ 1·109, $n_D^{15 \cdot 5}$ 1·514, $[\alpha]_D$ —100°, and was a pale yellow liquid which rapidly darkened and resinified on exposure to light and air (Found: C, 78·15, 77·9, 78·2, 77·7; H, 9·05, 9·0, 9·05, 9·3%. Calc. for $C_{15}H_{22}O_2$: C, 76·9; H, 9·5%. Calc. for $C_{16}H_{22}O_2$: C, 78·0; H, 9·0%).

TABLE 2

| Fraction | Mass (g) | Still-Head (°C) | $n_D^{20}$ | $[\alpha]_D$ (deg.) | $d_{15}^{15}$ |
|---|---|---|---|---|---|
| I | 3·5 | 104 | 1·5040 | — | |
| II | 3·0 | 112 | 1·5016 | — 8·6 | |
| III | 3·4 | 115 | 1·5018 | —13·5 | |
| IV | 3·35 | 117 | 1·5030 | —21·1 | |
| V | 3·5 | 120 | 1·5040 | —30·4 | |
| VI | 3·75 | 123 | 1·5010 | —34·8 | |
| VII | 3·7 | 125–128 | 1·4878 | —31·1 | |
| VIII | 3·0 | 124 | 1·4950 | —33·2 | |
| IX | 3·6 | 129 | 1·4862 | —34·5 | 1·023 |
| X* | 4·0 | 130 | 1·4833 | —34·5 | |
| XI | 4·0 | 130 | 1·4832 | —34·6 | 1·027 |
| XII | 3·7 | 130–131 | 1·4820 | —34·5 | |
| XIII | 3·6 | 131 | 1·4818 | —34·4 | |
| XIV† | 3·7 | 131 | 1·4813 | —34·4 | 1·030 |
| XV | 3·9 | 132 | 1·4812 | —33·2 | |
| XVI | 3·6 | 131–132 | 1·4818 | —31·8 | |
| XVII | 3·95 | 134 | 1·4826 | —30·0 | |
| XVIII | 4·4 | 135–140 | | | |

* Found: C, 72·1; H, 9·35%.

† Found: C, 71·95; H, 9·3%. Calc. for $C_{15}H_{24}O_3$: C, 71·4; H, 9·5%.

No solid semicarbazone or 2,4-dinitrophenylhydrazone could be obtained, but signs of reaction were observed. Ultraviolet absorption was observed at $\lambda_{max.}$ 230 mμ, log $\varepsilon_{max.}$ 3·89, $\lambda_{inflex.}$ 280 mμ, log $\varepsilon_{inflex.}$ 3·29. Hydrogenation with Adam's catalyst gave an oil, $d_{15 \cdot 5}^{15 \cdot 5}$ 0·[illegible]14, $[\alpha]_D$ +7·55, $n_D^{15 \cdot 5}$ 1·477 (Found: C, 75·8; H, 11·1%. Calc. for $C_{15}H_{26}O_2$: C, 75·6; H, 10·9%). A small amount of a 2,4-dinitrophenylhydrazone, m.p. 185–186 °C was obtained from this product.

Oxidation with potassium ferricyanide (Brandt and Ross 1949) gave 3-furoic acid (60 mg from 4 g), m.p. 120–121 °C, undepressed by an authentic specimen kindly provided by the late Dr. C. Brandt and *iso*butyric acid (*p*-bromophenacyl ester, m.p. 75 °C undepressed by an authentic specimen). There was no evidence of the presence of pyruvic acid.

Oxidation with potassium permanganate in acetone in the usual manner gave *iso*valeric acid (*p*-bromophenacyl ester, m.p. 65 °C undepressed by an authentic specimen) and two neutral solids. One of these was a hydrocarbon $C_nH_{2n+2}$ (*n* about 20), m.p. 57 °C, which must be an impurity and may partly explain the high analytical values of the total fraction although the amount isolated was small (80 mg from 10 g) (Found : C, 85·25 ; H, 14·7%. Calc. for $C_{20}H_{42}$ : C, 85·1 ; H, 14·9%). The other neutral solid had m.p. 171 °C (from alcohol). It appeared to give with difficulty a 2,4-dinitrophenylhydrazone, m.p. 122 °C, and in solution in tetrachloroethylene showed bands in the infra-red at 5·88 μ (C=O) ; 7·20 and 7·29 μ (equal intensities) ($C(CH_3)_2$) ; 3·06 and 2·9 μ (OH) (Found : C, 65·4, 66·3, 66·5 ; H, 7·7, 8·0, 8·1%. Mol. wt. (Rast) : 177, 190. Calc. for $C_{10}H_{14}O_3$ : C, 65·9 ; H, 7·7%. Mol. wt. (Rast) : 182. Calc. for $C_{13}H_{18}O_4$ : C, 66·0 ; H, 7·6%. Mol. wt. (Rast) : 238.

(*d*) *Fractionation of Oil Sample* (*B*)

TABLE 3

| Fraction | Mass (g) | Still-Head (°C) | $d^{15\cdot5}_{15\cdot5}$ | $n^{15\cdot5}_{D}$ | $[\alpha]_D$ (deg.) | $\lambda_{max.}$ (mμ) | $\varepsilon_{max.}$ |
|---|---|---|---|---|---|---|---|
| I | 2·2 | 109 | 0·971 | 1·5072 | —20 | | |
| II | 2·7 | 115–118 | 1·000 | 1·5111 | —36 } * | 279 | 700 |
| III | 2·1 | 115 | 0·998 | 1·5113 | —39 } | | |
| IV | 2·0 | 115 | | 1·5115 | —36 | | |
| V | 2·1 | 115 | 1·000 | 1·5113 | —35 | | |
| VI | 1·8 | 115–120 | | 1·5111 | —29 | | |
| VII | 2·2 | 117 | 0·998 | 1·5113 | —16 | | |
| VIII | 2·1 | 119–120 | | 1·5110 | —12† | | |
| IX | 2·3 | 120 | 1·000 | 1·5100 | + 4 | | |
| X | 2·3 | 121 | 1·010 | 1·5085 | +11‡ | 237 | 7100 |
| XI | 2·4 | 125 | 1·024 | 1·5052 | + 2 | | |
| XII | 2·4 | 121 | 1·031 | 1·5014 | —20 | | |
| XIII | 2·7 | 121 | 1·034 | 1·5000 | —35§ | 237 | 4800 |

* Found : C, 79·19 ; H, 9·73%.
† Found : C, 82·4 ; H, 11·0%. Calc. for $C_{15}H_{24}O$ : C, 81·8 ; H, 10·9%.
‡ Found : C, 75·06 ; H, 9·72%.
§ Found : C, 74·36 ; H, 8·89%.

## IV. Acknowledgments

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# THE SESQUITERPENE ALCOHOLS OF *EUCARYA SPICATA* SPRAGUE & SUMM.

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*Summary*

The anomalous properties of the santalol fraction of West Australian sandalwood oil have been explained by the detection of a considerable proportion of farnesol, which can be reduced by sodium and alcohol in liquid ammonia to dihydrofarnesene (I), and oxidized by manganese dioxide to farnesal.

## I. Introduction

Australian sandalwood oil from the heartwood of *Eucarya spicata* Sprague & Summ. (syn. *Santalum spicatum* R.Br.) (Stickwood) contains more than 90 per cent. of sesquiterpene alcohols, the nature of which has been the subject of some controversy. Rao and Sudborough (1923) originally reported the isolation of the secondary alcohols " α- and β-fusanol ", but there is much doubt as to their homogeneity. Penfold (1928) established the presence of α-santalol, and later of β-santalol (1932). Penfold and other workers (Watson 1925 ; May 1928 ; Simonsen 1931) observed that the sesquiterpene alcohol fractions possessed a lower refractive index, density, and optical rotation than would be expected for a mixture of α- and β-santalol. On occasion, specimens of alcohols of markedly low densities, namely, 0·9439 to 0·9486, were obtained from individual logs of a series supplied during the past 25 years by the Conservator of Forests for Western Australia. The primary alcohols were isolated with phthalic anhydride in the usual manner and distilled until a fraction of unaltered low density was obtained. The fraction used in this work had the following constants : b.p. 138–139 °C/2–3 mm, $d^{15}_{15}$ 0·9353, $[\alpha]_D$ ±0°, $n^{20}_D$ 1·5041. This may be compared with relatively pure santalols : α-, b.p. 166–167 °C/14 mm, $d^{25}_{25}$ 0·9770, $[\alpha]_{5461}$ +10·3°, $n^{25}_D$ 1·5017 ; β-, b.p. 177–178 °C/17 mm, $d^{25}_{25}$ 0·9717, $[\alpha]_{5461}$ −87·1°, $n^{25}_D$ 1·5100. Such unexplained variations in " constants " can be of considerable commercial importance.

The infra-red spectrum of the alcohol fraction was compared with that of a commercial mixture of α- and β-santalol from East Indian sandalwood oil, and it was found that many bands in the " finger-print " region 7–14μ coincide, although the spectra are not identical. In order to explain the physical constants of the alcohol mixture, the most obvious hypothesis was that it contains a marked proportion of an acyclic primary alcohol such as farnesol, b.p. 120 °C/0·3 mm, $d^{14}_8$ 0·8954, $[\alpha]_D$ 0°, $n^{20}_D$ 1·4877.

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The structure of the primary allylic sesquiterpene alcohol, lanceol, was largely determined by its reduction with sodium and ethanol in liquid ammonia to β-bisabolene; the santalols were also reduced to santalenes in the same way (Birch and Murray 1951). The sandalwood alcohol fraction was similarly treated and gave rise to a hydrocarbon mixture from which was obtained dihydrofarnesene trihydrochloride. This was identified by comparison with an authentic specimen derived from the reduction product of nerolidol. It is interesting to note that the primary alcohol is much less readily reduced than the tertiary, as in the comparable cases of geraniol and linalool (Birch 1950).

On this evidence the unidentified constituent must be farnesol or some other primary alcohol which gives rise to dihydrofarnesene. Preliminary ozonolysis experiment undertaken by Sir John Simonsen, F.R.S., in 1936 had demonstrated the formation of acetone and probably laevulinic aldehyde, together with a neutral ketone, possibly geranyl acetone, which could be derived from farnesol (I). Further experiments on a small quantity tended to confirm the formation of laevulinic aldehyde, isolated as its 2,4-dinitrophenylhydrazone. Attempts to prepare farnesal by oxidation with Beckmann's chromic acid mixture resulted only in the isolation and santalal semicarbazone, m.p. 230 °C. Artificial mixtures of santalols (45 per cent.) and farnesol (55 per cent.) having physical constants practically identical with the fraction also produced only santalal on oxidation. Farnesol was finally identified by oxidation with manganese dioxide in light petroleum solution (Weedon and Woods 1951). The product gave rise to farnesal semicarbazone, m.p. 128–130 °C.

This oxidation process is specific for allyl alcohols and probably proceeds through a mesomeric radical (II). The extra methyl group on an end carbon atom of II compared with the corresponding radical (III) from the santalols would render its formation easier, and may explain the exclusive formation of farnesal when only partial oxidation was carried out.

$$\text{(I)}\quad (CH_3)_2C{=}CH\;CH_2CH_2C(CH_3){=}CH\;CH_2CH_2C(CH_3){=}CH\;CH_2OH$$

$$\text{(II)}\quad C_{11}H_{19}\underbrace{\overset{\overset{\displaystyle CH_3}{|}}{C}\cdots CH\cdots CHOH}_{*}$$

$$\text{(III)}\quad C_{11}H_{17}\underbrace{CH\cdots\overset{\overset{\displaystyle CH_3}{|}}{C}\cdots CHOH}_{*}$$

## II. Experimental

(*a*) *Isolation*.—The following run is typical of the results obtained with *E. spicata* sawdust and shavings. The material (82 lb) was distilled for 18 hr at a steam-pressure of 10 lb/sq. in. to yield 2% of oil containing 90% alcohols estimated as $C_{15}H_{24}O$. The oil had the following characteristics: $d_{15}^{15}$ 0·9504, $n_D^{20}$ 1·5040, $[\alpha]_D^{20}$ +0·5°, ester value 17, after acetylation 200.

This material was derived from a commercial stand on rich flats ; the area has since been denuded. The primary alcohol fraction which is the major portion of the oil was obtained by treatment with phthalic anhydride in benzene in the usual manner, and fractionated to give a product with the constants already noted.

(*b*) *Reduction.*—A solution of the sesquiterpene alcohol (3 g) in ethanol (12 c.c.) was added to the liquid ammonia (200 c.c.) with stirring, followed by potassium (12 g) slowly and in small pieces. After the blue colour had disappeared (about 3 hr), water (100 c.c.) was cautiously added, the oil taken up in ether and distilled: (i) b.p. 90–112 °C/0·5 mm (0·5 g); (ii) b.p. 112–122 °C/0·5 mm (0·7 g); (iii) b.p. 122–143 °C/0·35 mm (1·3 g). The reduction was evidently incomplete, and the higher fractions were again reduced, and the whole distilled over sodium to give a hydrocarbon fraction, b.p. 82–88 °C/0·5 mm (1·6 g). This fraction (1 g) in ether (4 c.c.) was saturated with dry hydrogen chloride and left at 0 °C for 45 min. The solvent was removed under reduced pressure, methanol (2·5 c.c.) was added to the residue, and after leaving in the refrigerator white plates separated from the oil. After draining on a porous tile and crystallization from methanol, colourless plates of dihydrofarnesene trihydrochloride were obtained, m.p. 52 °C undepressed by the derivative below (Found: Cl, 32·9%. Calc. for $C_{15}H_{19}Cl_3$: Cl, 33·8%).

(*c*) *Reduction of Nerolidol.*—Nerolidol (5 g) in ethanol (20 c.c.) and liquid ammonia (350 c.c.) was reduced by the action of sodium (16 g) during 4 hr. Distillation of the product over sodium gave a main fraction, b.p. 86–88 °C/1 mm (3 g). This gave rise in good yield to dihydrofarnesene trihydrochloride, m.p. 52 °C when treated as above. Farmer and Sutton (1942) give m.p. 52 °C for this derivative.

(*d*) *Ozonolysis.*—(i) (Preliminary work carried out by Sir John L. Simonsen, F.R.S., in 1936.) The sesquiterpene alcohol fraction (2·3 g) in methyl acetate (20 c.c.) was ozonized at 0 °C, the issuing gases being passed through water. Only a trace of formaldehyde dimedone derivative could be obtained from the water. The methyl acetate was removed under reduced pressure, the gum mixed with water (10 c.c.), and heated on the steam-bath, the volatile liquid being trapped in 2,4-dinitrophenylhydrazine sulphate solution. A derivative was so obtained, crystallizing from ethanol, m.p. 122 °C, undepressed by an authentic specimen of the acetone derivative, m.p. 122–124 °C. The aqueous solution (A) was extracted with ether, the extract washed with sodium hydroxide (B), and the insoluble oil so obtained reacted with semicarbazide and with phenylsemicarbazide to give a gum. After recovery from the latter derivative by steam distillation in the presence of oxalic acid an oil was obtained which produced a 2,4-dinitrophenylhydrazone as yellow needles, m.p. 81–83 °C (Found: C, 60·9; H, 5·66; N, 15·2%. Calc. for $C_{18}H_{20}O_4N_4$: C, 60·7; H, 5·6; N, 15·7%). The nature of this product is not clear; it may be the derivative of geranylacetone (Calc. for $C_{19}H_{26}O_4N_4$: C, 60·9; H, 6·9; N, 15·0%).

The alkaline extract (B) yielded a liquid acid (0·6 g) which reacted with carbonyl reagents and gave the bromoform reaction; it is probably laevulinic acid. The original aqueous solution A from the ozonide breakdown, which had been extracted with ether, gave a very sparingly soluble 2,4-dinitrophenylhydrazone (probably a bis-derivative), m.p. *c.* 300 °C, which may be the osazone of hydroxyacetone.

(ii) In order to amplify some of the results of the foregoing preliminary investigation, the ozonolysis was repeated.

(1) The alcohol fraction (0·5 g) in ethyl acetate (10 c.c.) at 0 °C was submitted to the action of ozonized oxygen in a fairly rapid stream for 60 min. The ozonide was decomposed with acetic acid (80%; 8 c.c.) and zinc dust (0·2 g) and filtered. To the filtrate was added 2,4-dinitrophenylhydrazine in 2N hydrochloric acid, the derivative taken up in benzene and chromatographed twice on an alumina column (*c.* 25 g). Arbitrary division of the eluate into fractions gave only one crystalline compound as orange needles from m.p. 127 °C. The 2,4-dinitrophenylhydrazone of hydroxyacetone has m.p. 129 °C (Strain 1935), but was not available for comparison and there was insufficient material for analysis.

(2) The alcohol (1 g) was ozonized as before but for 6 hr. Steam distillation (about 500 c.c. of distillate) gave a series of fractions (50 c.c.) which all reacted with 2,4-dinitrophenylhydrazine. The derivative from the first three fractions, after crystallization from ethyl acetate formed

orange needles, m.p. 246–246·5 °C (Found : C, 41·8 ; H, 3·6%. Calc. for $C_8H_8O_5N_4$ : C, 42·8 ; H, 3·6%). It may therefore be the derivative of glyoxal. The next three fractions were converted to the 2,4-dinitrophenylhydrazone, which was chromatographed on alumina in ethyl acetate, and then crystallized from chloroform as dark orange needles, m.p. 231–232 °C, rather insoluble in ethanol, and probably a bis-derivative (Found : C, 43·7 ; H, 3·4%. Calc. for $C_{17}H_{16}O_8N_8$ : C, 44·3 ; H, 3·4%). The ultraviolet absorption at $\lambda_{max.}$ 358 mμ, $\varepsilon_{max.}$ 36,376 (in chloroform) indicated that the carbonyl groups of the original substance could not be adjacent, and that neither is αβ- to a double bond. The substance is probably the bis-2,4-dinitrophenylhydrazone of laevulinic aldehyde, m.p. 231 °C (Wilson 1948).

The remaining four portions of 50 c.c. gave a pale yellow 2,4-dinitrophenylhydrazone, m.p. 196 °C, after treatment as above and chromatography on alumina in ethyl acetate (Found : N, 22·5%). Insufficient material was available for further investigation.

(e) *Farnesal*.—(i) Farnesol (pure 500 mg) was shaken for 3 hr with precipitated manganese dioxide (5 g dried at 100 °C) and light petroleum (15 c.c. ; b.p. 40–70 °C) and then left for 48 hr. The product was then reacted with semicarbazide acetate in the usual manner and chromatographed on alumina (15 g). Elution with light petroleum (b.p. 40–70 °C) brought some unchanged farnesol through the column ; methanol then eluted the semicarbazone. This was crystallized first from light petroleum (b.p. 40–70 °C) and then from aqueous methanol, forming matted needles, m.p. 128–130 °C.

(ii) A repetition of the process above with sesquiterpene alcohol fraction (1 g) gave rise to the same product, m.p. 127–130 °C, undepressed by the authentic derivative. The oxidation in this case was very incomplete, and a considerable proportion of starting-material was recovered.

## III. Acknowledgments

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# THE REDOX PROPERTIES OF NICOTINAMIDE METHOHALIDES

By S. J. Leach,* J. H. Baxendale,† and The Late M. G. Evans‡

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*Summary*

The reversible reduction of nicotinamide methiodide and methochloride has been studied using cathodic reduction at a mercury surface, sodium dithionite, hydrogen and colloidal platinum, and various leuco-dyes. The method of cathodic reduction was the most satisfactory. Polarography showed a 2-step reduction. Macro-reductions at a mercury surface suggested dimerization of the intermediate free radical. The behaviour of dihydromethylnicotinamide towards hydrogen ions, oxygen, iodine, potassium ferricyanide, ferric iron, and various dyes has been examined. Although it is not readily oxidized by oxygen it is capable of reducing most other systems with a higher $E_0'$. Two methods of estimating the reduced compound are suggested and involve (*a*) the potentiometric titration of potassium ferricyanide at pH 9·1 and (*b*) the reduction and decolourization of 2,6-dichlorophenolindophenol at pH 4·7. The oxidation-reduction potential of nicotinamide methiodide has been measured by oxidative titration of the dihydro-compound at pH 9·1 and 30±0·01 °C and found to be —0·36±0·02 V against the normal hydrogen electrode. The titration curve does not show a separation of the two reduction steps. Evidence is discussed for the production of both *o*- and *p*-dihydromethylnicotinamide during reduction.

## I. Introduction

Since elucidation of the composition of coenzyme II (Warburg and Christian 1934–35) and of coenzyme I (Schlenk and Euler 1936) there has been interest in the properties of nicotinamide methohalides as simple structural analogues. Co I has a limited specificity as a coenzyme, being capable of promoting the oxidation or reduction of some 14 pairs of substrates each requiring a highly specific protein (Negelein and Wulff 1937 ; Warburg and Christian 1939 ; Kubowitz and Ott 1943). Furthermore there is evidence that substances other than Co I may act as the prosthetic group in three of these systems (Dixon and Zerfas 1940). Against this, the complicated structure of Co I may not be much modified without interfering with its specificity (Schlenk, Hellström, and Euler 1938 ; Schlenk 1945). It is therefore important to determine the relative importance of redox potential, structure, and oxidation mechanism in deciding which substrates may be attacked.



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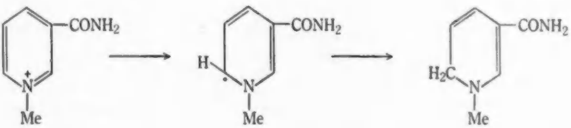
Those properties of the coenzyme which may be looked for in the model coenzyme are: (1) its ability to reduce in two steps with the formation of an intermediate radical (Adler, Hellström, and Euler 1936; Hellström 1937; Drabkin 1945); synthetic analogues are already known to show this property qualitatively (Karrer and Benz 1936; Schlenk 1945); (2) its extremely low redox potential of $-0 \cdot 320$ V against the normal hydrogen electrode (N.H.E.) (Burton 1952) at pH 7, enabling it slowly to reduce various dyes with more positive redox potentials; and (3) its inability to react rapidly in the reduced state with molecular oxygen. The last two properties appear to be inconsistent with one another unless the mechanism of reduction is as important as the redox level. Similar conclusions have been reached using other systems (Green, Stickland, and Tarr 1934; Dickens 1936; Michaelis and Smythe 1936; Dickens and McIlwain 1938).

Karrer *et al.* (1936) have reported a series of extremely negative $E_0'$ values at various pH's for nicotinamide methiodide. However, they were obtained with unpoised fully reduced systems from the potentials of a single electrode immersed for 24 hr. The same objection applies to measurements on other compounds (Karrer 1937; Karrer, Schwarzenbach, and Utzinger 1937; Karrer and Stare 1937; Karrer, Ishii *et al.* 1938; Karrer, Kahnt *et al.* 1938) in this series. Michaelis and Smythe (1938) have reported that unpublished experiments on the methyl compound were " unsatisfactory but they at least suggest a very negative potential ".

The purpose of the present investigations was to determine accurately the redox potential of nicotinamide methohalides and to examine their reduction and subsequent oxidation by other redox systems. By using potentiometry and polarography it was also intended to seek evidence of " semiquinone " formation of the following type:



## II. Experimental

(*a*) *Preparation and Estimation of Nicotinamide Methiodide and Methochloride.*—With minor modifications the methods used were those of Karrer and Benz (1936). The iodide was estimated by titration with $AgNO_3$ in the presence of eosin and was found to be 97–98% pure. Solutions of the iodide were prepared only as required. The chloride was prepared from the iodide by shaking with AgCl.

(*b*) *Purification of Rosinduline 2G* (*Colour Index No. 830*).—The commercial product was purified by repeated precipitation from alkaline solution by the addition of acid. Its purity was estimated by $K_3Fe(CN)_6$ titration of the substance reduced with $H_2$ and colloidal Pt at pH 9·1. At this pH the $E_0'$ was found to be $-0 \cdot 394$ V against N.H.E. compared with $-0 \cdot 395$ V quoted by Michaelis (1931), and its purity was 98·5%.

(*c*) *Preparation of Methyl Viologen.*—Methochloride (0·4 g) was prepared from γ–γ'-dipyridyl (1·5 g) by the method of Michaelis (1933).

(*d*) *Purification of Phenosafranine* (*Colour Index No. 840*).—The commercial dye was purified in the manner suggested by Stiehler, Chen, and Clark (1933).

(*e*) *Preparation of Colloidal Platinum.*—Gum arabic (0·5 g) was dissolved in hot water (1 l.) and $H_2PtCl_6$ (0·5 g) and 50% $N_2H_4.H_2O$ (0·1 ml) added to the cooled solution. The colloid obtained by dialysis for several days contained Pt (0·3 g/l.) and retained its catalytic efficiency for 2–3 wk when stored at 0 °C.

(*f*) *The Reduction of Nicotinamide Methohalides.*—For measurements of redox potential, solutions were required containing $10^{-4}$ to $10^{-2}$M dihydromethylnicotinamide and owing to the slow reoxidation of the product the solutions had to be freshly prepared. The use of $Na_2S_2O_4$ for reduction (Karrer and Benz 1936) produces an oily product containing inorganic contaminants while $H_2$ and colloidal Pt may give irreversible reduction to the hexahydro-compound. Both of these methods were re-examined in the present work before proceeding to the more satisfactory method of cathodic reduction. No by-products are formed here and a substance may be reduced to any desired " level " by maintaining a constant cathode potential. The method has not previously been applied to nicotinamide methochloride and in order consistently to reduce the substance to a predetermined level it was first necessary to determine the cathode potentials required using polarography as a pilot technique.

(i) *Polarographic Reduction.* Nicotinamide methiodide in $2\times10^{-3}$M buffer solutions were used in a Cambridge recording polarograph. HCl and buffers containing glycine and phosphate were used to cover pH's from 2·03 to 9·0 and polarograms were obtained in an atmosphere of $H_2$ with no attempt to suppress maxima.

(ii) *Cathodic Reduction on a Macro-Scale.* The apparatus was a modification of that used by Lingane, Swain, and Fields (1943) and Lingane (1947) for reduction of an acridine derivative. The 250 ml conical cathode compartment contained a mercury pool of surface area 64 sq. cm. and the anode mercury had an area of 9·6 sq. cm. Between the two compartments a porous plate and a plug of agar-agar/KCl prevented mass-movement and the plug was renewed before each reduction. The anolyte was usually 0·1N NaOH or an alkaline buffer and provision was made for outgassing both this solution and the catholyte with purified nitrogen. By carrying the outgassing tube down to the surface of the mercury anode, concentration polarization was prevented during electrolysis. A rapid stirrer fitted with a mercury seal helped in the outgassing of the catholyte and ensured adequate mixing during reduction.

Current was supplied from accumulators connected across a 1290 Ω rheostat and a suitable e.m.f. was tapped off and applied across the electrolytic cell. The cathode potential was measured against a saturated calomel half-cell by means of a potentiometer, and was kept constant by periodically diminishing the e.m.f. across the cell. The current through the cell was recorded manually and fell to a small constant value in 30–60 min.

To transfer the electrolysed solution to the titration vessel quantitatively in the absence of air, it was blown by $N_2$ gas into a $N_2$ filled calibrated 100 ml bulb and then allowed to drain into the vessel.

(*g*) *Potentiometric Titration.*—The progress of oxidation of dihydromethylnicotinamide during titration was followed potentiometrically in an atmosphere of $N_2$. Nitrogen was purified by passage through four successive towers of alkaline $Na_2S_2O_4$ containing indigodisulphonate (Stevens *et al.* 1945) followed by a copper tower (Meyer and Ronge 1939) at 200 °C. The gas was then passed through 10% KOH, 5% $HgCl_2$, and finally through distilled water. It was used for outgassing either the titration cell or the titrant vessel.

The titration apparatus is shown in Figure 1. The 120 ml electrode vessel thermostated at 30±0·01 °C carried four electrodes (two Au and two Pt) and a KCl bridge. The purified $N_2$ was introduced through a sintered-glass bubbler, and served to stir the solution after each addition of titrant. Electrode potentials were measured against a saturated calomel electrode (S.C.E.) which was maintained at the thermostat temperature. The potentiometer was a Cambridge slide-wire instrument reading to 0·2 mV which was used with a Tinsley galvanometer af 500 Ω resistance and a sensitivity of 29·5 mm/μA. A 1000 Ω resistance was used in series with the galvanometer except near the balance point. The apparatus was tested by reductive titration

of methyl viologen and rosinduline 2G with $Na_2S_2O_4$ solution. Electrode agreement was within 0·1 mV for 3 of the electrodes and the $E_0$'s obtained were in good agreement with the literature, for example, rosinduline 2G at pH 9·05 and 30 °C gave —0·635 V against S.C.E.

(i) *Estimation of Dihydromethylnicotinamide.*—After electrolytic reduction the dihydromethylnicotinamide was forced by gas pressure into the titrant flask through an all-glass connection, where it was thoroughly outgassed with $N_2$. This was necessary to remove $H_2$ which sometimes interfered with the electrical stability of the electrode. The $10^{-4}$M $K_3Fe(CN)_6$ was dissolved in glycine buffer of pH 9·1 and outgassed for 30 min. 1 ml portions of $10^{-3}$M dihydromethylnicotinamide were added and oxidation proceeded extremely rapidly with electrode agreement to 0·1 mV. End-points were estimated to <0·1 ml. A second method of estimation involved the reduction of 2,6-dichlorophenolindophenol at pH 4·7. Dihydromethylnicotinamide was pipetted into excess ($8\times10^{-4}$ to $9\times10^{-5}$M) dye to produce an immediate reduction in colour followed by a very slow fading. Fading interfered with the colorimetric assay when "aged" solutions of dihydromethylnicotinamide were used, but with fresh dihydromethylnicotinamide the method gave rapid and reproducible results which compared well with the potentiometric method.

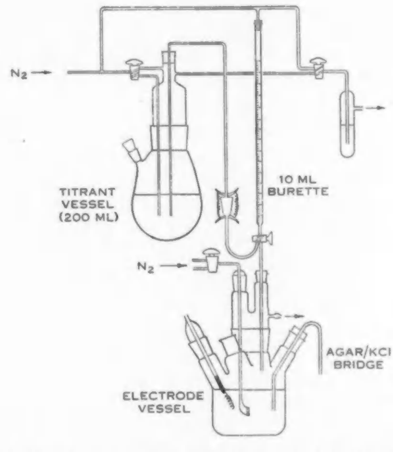



**Fig. 1.—Apparatus for potentiometric titration in an atmosphere of nitrogen.**

(j) *The Oxidative Titration of Dihydromethylnicotinamide.*—The results quoted for the redox potential of dihydronicotinamide were obtained by titration with $K_3Fe(CN)_6$ in the presence of rosinduline 2G as a potential mediator. A typical experiment is described here :

> Nicotinamide methiodide ($2\times10^{-3}$M ; 200 ml) was reduced electrolytically in glycine buffer (pH 9·05) at a cathode potential of —1·7 to —1·8 V against S.C.E. The current passing was 80% of the theoretical for a 2-electron transfer. 100 ml was immediately transferred by $H_2$ pressure to the outgassed titration vessel and $H_2$ passed through via the sintered bubbler. A half-immersed platinized Pt electrode showed an $E_h$ of —0·5433 V corresponding to pH 9·05 at 30 °C. This value corresponded closely with the pH value found in the absence of dihydromethylnicotinamide. The $H_2$ electrode was removed, the solution outgassed with $N_2$ and 5 ml of $2\times10^{-3}$M rosinduline 2G in outgassed aqueous solution were added. Titration with $2\times10^{-2}$M $K_3Fe(CN)_6$ gave the results shown in Table 4. A very slow stream of $N_2$ was maintained during measurement and the potentials recorded by the Pt spiral were used in subsequent calculations as being the more stable.

## III. Results

### (a) *The Reduction of Nicotinamide Methiodide*

(i) *Reduction by $Na_2S_2O_4$.*—The slow addition of $10^{-2}$M $Na_2S_2O_4$ in glycine buffer of pH 9·1 to $5\times10^{-2}$M nicotinamide methiodide in the same buffer was followed potentiometrically and resulted in slow reduction. Electrode potentials drifted down to −0·41 V against N.H.E. and were too unstable for accurate titration. In other experiments excess $Na_2S_2O_4$ was added to the methiodide solution and left to react for 2 hr. Using rosinduline 2G as a potential mediator the solutions were titrated with 0·5M $K_3Fe(CN)_6$ and a plot of electrode potential against ml titrant showed that 60 per cent. of the nicotinamide methiodide had been reduced. Approximate $E_0'$ values were usually −0·34 V against N.H.E.

(ii) *Reduction by $H_2$ and Colloidal Pt.*—$H_2$ was passed into a $10^{-3}$M solution of nicotinamide methiodide in glycine buffer at pH 9·1 containing 5 ml of Pt colloid and $5\times10^{-5}$M rosinduline 2G. Although it has been reported that the hexahydro-compound is produced with $H_2$ and colloidal Pt, under the conditions used in the present work some dihydro-compound was formed. The rate of reduction was low and even after 3 hr oxidative titration of the solution showed that only 20 per cent. reduction had occurred. A good titration curve was obtained with electrode agreement to 0·1 mV and the $E_0'$ was −0·377 V against N.H.E.

Table 1

Values of $E_{\frac{1}{2}}$ for $2\times10^{-3}$M solutions of nicotinamide methiodide

| Buffer | pH | $E_{\frac{1}{2}}$ against S.C.E. | |
|---|---|---|---|
| | | 1st Step | 2nd Step |
| Glycine .. .. | 9·0 | −0·94 | −1·57 |
| Phosphate .. .. | 7·0 | −0·94 | ∼−1·50 |
| Phosphate .. .. | 5·15 | −0·94 | −1·32 |
| Hydrochloric acid | 2·03 | ∼−0·9 | Obscured |

(iii) *Reduction of Leuco-Dyes.*—Methyl viologen ($5\times10^{-4}$M), which had been reduced by $Na_2S_2O_4$, attacked $5\times10^{-3}$M nicotinamide methiodide only slowly. Rosinduline 2G, reduced by $H_2$ and colloidal Pt was titrated with the methiodide in $10^{-3}$M concentration but there was no interaction. The potential of the dye showed no increase and its colour remained pale yellow.

(iv) *Polarographic Reduction.*—In a series of polarograms at pH's between 5·15 and 9·0 and at concentrations of $2\times10^{-3}$M the reduction proceeded in two distinct steps. A curve obtained at pH 2·0 showed a sudden and continuous increase in current commencing at a cathode potential of −0·80 V due to $H_2$ liberation. Table 1 gives the values of $E_{\frac{1}{2}}$ obtained.

The first reduction step is independent of pH but the second is not. This is in accordance with the observations of Tompkins and Schmidt (1943) although their potentials were about 0·1 V more negative throughout. As in the present study the slope and $E_{\frac{1}{2}}$ of the second reduction wave varied with the buffer used.

(v) *Cathodic Reduction.*—Before reduction the cathode potential was usually —0·10 to —0·15 V and after reduction (when the applied e.m.f. was discontinued) it was usually —1·0 to —1·1 V. A typical plot of the current-time curve is shown in Figure 2 in which the cathode potential was maintained constant at —1·7 to —1·8 V. For this reduction the methiodide was $5 \times 10^{-3}$M

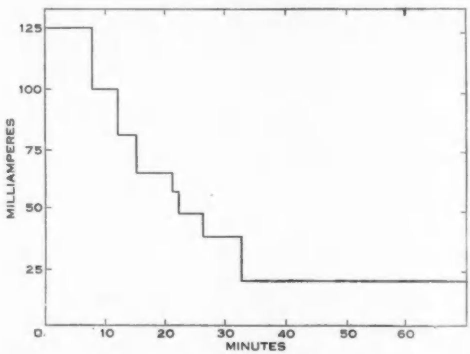
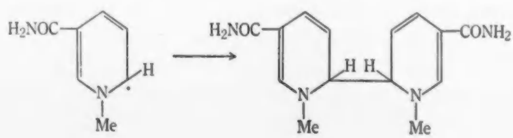



Fig. 2.—Typical current-time curve for the cathodic reduction of $5 \times 10^{-3}$M nicotinamide methiodide at a cathode potential of —1·7 to —1·8 V.

and the total quantity of current passed was 106 C. In other two reductions at the same cathode potential, concentrations of $2 \times 10^{-3}$M and $1 \times 10^{-3}$M were used and the quantity of current passed was 55 and 31 C respectively. Assuming a 2-electron transfer the percentages of the theoretical amounts of current passed for complete reduction were 53·0, 69·6, and 77·5 per cent. for these three cases. In all, 35 cathodic reductions were carried out. The average value of the "current efficiency" for 16 determinations in $2 \times 10^{-3}$M solutions at pH 9 and using a cathode potential of —1·7 to —1·8 V was 78·5 per cent. with actual reductions varying from 73 to 84 per cent. efficiency. Higher concentrations gave markedly lower efficiencies (about 50–60 per cent.). This concentration effect has not been pursued further but may be due to a dimerization of the half-reduced nicotinamide competing with the second stage of reduction,



Dimerization at the mercury electrode has been suggested by Tompkins and Schmidt (1943). The presence of dimer has not been proved but it was observed that even after prolonged storage, when the reducing power of the solution had virtually disappeared, the pale green colour of the solution remained and was due to a non-reducing component.

The extent of reduction at lower cathode potentials, corresponding to only the first reduction step, was usually a little lower than the values quoted above. Using potentials of $-1 \cdot 3$ to $-1 \cdot 4$ V and concentrations of $2 \times 10^{-3}$M at pH 9·1 the average current efficiency for six determinations was 61·5 per cent. with actual efficiencies varying from 58·4 to 64·6 per cent.

Using the methods of estimation already described, the percentage of dihydromethylnicotinamide immediately after electrolysis was found to be consistently lower than that expected to be produced by the current passed. Examples of this discrepancy are shown in Table 2. The discrepancy suggests

TABLE 2

COMPARISON OF CURRENT EFFICIENCY AND REDUCING POWER AFTER CATHODIC REDUCTION OF NICOTINAMIDE METHIODIDE IN GLYCINE BUFFER OF pH 9·1

| Concentration ($M \times 10^{+3}$) | Cathode Potential against S.C.E. (V) | Current Efficiency* (%) | Reducing Power* (%) |
|---|---|---|---|
| 2 | —1·20/—1·35 | 62 | 40 |
| 2 | <1·45 | 63 | 45 |
| 2 | —1·7/—1·8 | 80 | 55 |
| 5 | —1·7/—1·8 | 53 | ~50 |
| 1 | —1·7/—1·8 | 77·5 | 50 |

* Assuming 2-electron reduction.

that although current was passed until a constant minimum was reached, the electrolysed solutions contained some oxidized compound. An analysis of the potentiometric titration curves showed that this was due partly to incomplete reduction and partly to reoxidation after electrolyses, as will be discussed later in this paper.

### (b) *The Reactivity of Dihydromethylnicotinamide*

(i) *Reactivity to $H^+$ Ions.*—Samples of electrolytically reduced dihydromethylnicotinamide at pH 9 were stored over $N_2$ and periodically estimated by titration of $K_3Fe(CN)_6$. The results showed a slow decomposition which was independent of the presence of $O_2$. When the solution was reduced and stored at pH 10·4 the decomposition was more rapid : the rates for both pH's are shown in Table 3. Electrolyses at pH 7 produced solutions which contained only a few per cent. of dihydromethylnicotinamide even on immediate estimation. At pH 5·15 no reduction product was found by titration of $K_3Fe(CN)_6$ but the pale yellow solution very slowly reduced 2,6-dichlorophenolindophenol. Addition

of $Na_2S_2O_4$ to the solution produced none of the deep yellow colour characteristic of the dihydro-compound.

(ii) *Reactivity to Air and $O_2$*.—The slow " aging " of dihydromethylnicotinamide was not accelerated in the presence of air or $O_2$. After passing $O_2$ through the reduced solution at 30 °C for 2 hr, estimation by titration of $K_3Fe(CN)_6$ showed no loss in reducing power (seven estimations during aeration gave a value of $0\cdot65 \pm 0\cdot01 \times 10^{-3}$M). On the other hand, aerobic oxidation took place very rapidly in the presence of platinized asbestos. When small amounts of 2,6-dichlorophenolindophenol or hydroquinone were added oxidation took place at a measurable rate : using 0·492M dihydromethylnicotinamide and $2\cdot176 \times 10^{-5}$M dye, aeration at 30 °C and pH 9·1 reduced the dihydromethylnicotinamide

TABLE 3

RATE OF DECOMPOSITION OF REDUCED METHYLNICOTINAMIDE STORED UNDER NITROGEN AT ROOM TEMPERATURE

| Buffer | Duration of Storage (hr) | Reduced Compound Found* (%) |
|---|---|---|
| Glycine pH 9·0 | 2 | 56·8 |
| | 5 | 47·2 |
| | 21·5 | 37·3 |
| | 29·5 | 35·2 |
| | 48 | 23·1 |
| Carbonate/ bicarbonate pH 10·4 | 0·5 | 47·7 |
| | 18 | 35·6 |
| | 42 | 24·0 |
| | 66 | 14·9 |

* Based on original methiodide.

concentration to 0·250M in 98 min. During the catalysed reaction $H_2O_2$ was produced and was estimated colorimetrically as pertitanic acid. Its amount corresponded to the amount of dihydromethylnicotinamide disappearing. The catalysed oxidation was zero order with respect to dihydromethylnicotinamide until near the end of reaction when the blue colour of the oxidized indophenol began to appear. Aerobic oxidation in the presence of hydroquinone at pH 9 and 30 °C gave rates which were not zero order in dihydromethylnicotinamide nor simple first order in catalyst. Again the catalyst did not appear in the yellow oxidized form until the reaction was nearly complete. Similar results were obtained with hydroquinone at pH 10·4.

(iii) *Reactivity to $I_2$*.—Known amounts of dihydromethylnicotinamide were added to an excess of $10^{-3}$M iodine in bicarbonate solution and the unreduced iodine estimated by titration with $As_2O_3$ in the presence of chloroform. The results were corrected for the slow attack of iodine on the oxidized form of nicotinamide methiodide but still showed extraordinarily high consumptions

of iodine corresponding to approximately 1·11 equiv of nicotinamide methiodide before reduction. This suggested that apart from reversible oxidation there was also substitutive attack in the pyridine ring.

(iv) *Reactivity to $K_3Fe(CN)_6$.*—In the potentiometric estimation of dihydromethylnicotinamide it was shown that $K_3Fe(CN)_6$ was a rapid oxidant. However, when *excess* $K_3Fe(CN)_6$ was added to dihydromethylnicotinamide the first rapid reduction to $K_4Fe(CN)_6$ was followed by a slow reaction which in 40 min could amount to several per cent. of the total $K_3Fe(CN)_6$ consumption. (Excess $K_3Fe(CN)_6$ was estimated iodometrically and also independently by titration with $Ce(SO_4)_2$: in the latter method methochloride was used rather than methiodide.)

Excess $K_3Fe(CN)_6$ was found to have no effect on unreduced methochloride or on glycine buffer, and the slow secondary reduction of $K_3Fe(CN)_6$ was thus apparently due to a weakly reducing component. This latter component was found to be rapidly oxidized by $Ce(SO_4)_2$. At the low concentrations used in potentiometry and in the absence of excess $K_3Fe(CN)_6$ the secondary reaction was unnoticeable.

(v) *Reactivity to $Fe^{+++}$ Ions.*—Dihydromethylnicotinamide was found to reduce $Fe^{+++}$ ions at pH's from 3 to 9. When dihydromethylnicotinamide was pipetted into excess $(NH_4)_2SO_4.Fe_2(SO_4)_3.24H_2O$ containing NaF and *o*-phenanthroline and adjusted to pH 3–4 with HCl, the $Fe^{++}$ ions immediately formed gave a red colour with the *o*-phenanthroline. The reduction was usually only about 60 per cent. quantitative due to destruction of dihydromethylnicotinamide at this pH. Reductions of ferricitrate or ferritartrate by dihydromethylnicotinamide at pH 9 were very slow: at pH 7 the reduction was more rapid and reaction was quantitative, agreeing well with the $K_3Fe(CN)_6$ estimation of dihydromethylnicotinamide.

(vi) *Reactivity to Dyes.*—Various dyes were titrated with dihydromethylnicotinamide at pH's 5·15 and 9·1 in phthalate and glycine buffer respectively and the reductions were followed potentiometrically. Methylene blue, 2,6-dichlorophenolindophenol, indigo-carmine, and benzoquinone were all rapidly reduced. Even when both reactants were $10^{-5}$M, reduction was complete in 5–10 sec at pH 9. The rates of reduction, however, were pH dependent. The indophenol gave excellent titration curves at both pH's but reduction was much more rapid at the lower pH. The reduction of this dye to the leuco-form in pH 4·7 acetate buffer was made the basis of a colorimetric method for estimating dihydromethylnicotinamide in the presence of air. Sodium anthraquinone-2-sulphonate showed no reduction in acid solution but was reduced rapidly at pH 9·1. Malachite green was not reduced with a 10-fold excess of dihydromethylnicotinamide in either neutral or alkaline solution. On the other hand the oxidized forms of xylene cyanol FF, erio green, methyl orange, and methyl red were easily reduced by dihydromethylnicotinamide.

### (c) *The Oxidation-Reduction Potential of Methylnicotinamide*

Without a potential mediator present no titration curve could be obtained since all potentials were unstable at −0·60 to −0·64 V against S.C.E. This

was true for both oxidative and reductive titration, and whether $Na_2S_2O_4$ or electrolysis was used as the means of reduction. Of the mediators used, rosinduline 2G was the most satisfactory. The most reproducible curves obtained were those involving the titration of cathodically reduced methylnicotinamide with $K_3Fe(CN)_6$ in the presence of rosinduline 2G.

In all, 15 oxidative titrations were carried out but the results for the typical experiment of Table 4 will be chosen for detailed description. Electrode agreement was rarely better than 2 mV and potentials varied by several mV

TABLE 4

POTENTIOMETRIC TITRATION OF $1 \cdot 156 \times 10^{-3}$M REDUCED METHYLNICOTINAMIDE WITH $2 \times 10^{-2}$M $K_3Fe(CN)_6$ AT pH 9·1 IN PRESENCE OF $9 \cdot 52 \times 10^{-5}$M ROSINDULINE 2G

| Titres (ml) | Electrode Potentials (V against S.C.E.) | | Oxidized Rosinduline (% calc.) | Corrections to Titre (ml ; $2 \times 10^{-2}$M $K_3Fe(CN)_6$) | Corrected Titres (ml) | Electrode Potentials (—V calc. from eqn. (1)) |
|---|---|---|---|---|---|---|
| | Platinum Plate | Platinum Spiral | | | | |
| 0·23 | 0·633 | 0·619 | 78·2 | 0·22 | 0·45 | 0·616 |
| 0·76 | 0·628 | 0·615 | 82·2 | 0·18 | 0·94 | 0·614 |
| 1·43 | 0·623 | 0·611 | 85·9 | 0·14 | 1·57 | 0·611 |
| 2·23 | 0·618 | 0·6075 | 88·7 | 0·11 | 2·34 | 0·6082 |
| 3·23 | 0·614 | 0·6045 | 90·5 | 0·10 | 3·33 | 0·6045 |
| 4·23 | 0·611 | 0·6010 | 92·3 | 0·08 | 4·31 | 0·6010 |
| 5·23 | 0·607 | 0·5975 | 93·7 | 0·06 | 5·29 | 0·5975 |
| 6·23 | 0·603 | 0·5937 | 94·7 | 0·05 | 6·28 | 0·5938 |
| 7·23 | 0·599 | 0·5890 | 95·8 | 0·04 | 7·27 | 0·5898 |
| 8·23 | 0·594 | 0·5843 | 96·7 | 0·03 | 8·26 | 0·5851 |
| 9·24 | 0·589 | 0·5795 | 97·4 | 0·03 | 9·27 | 0·5793 |
| 10·25 | 0·581 | 0·5715 | 98·0 | 0·02 | 10·27 | 0·5708 |
| 10·75 | 0·575 | 0·5655 | 98·4 | 0·02 | 10·77 | 0·5639 |
| 11·15 | 0·569 | 0·5587 | 98·7 | 0·01 | 11·16 | 0·5547 |

with the rate of bubbling of the $N_2$. No such effects were observed with simple inorganic or dye systems. Owing to the addition of 5 per cent. rosinduline 2G some of the dihydromethylnicotinamide was oxidized before titration commenced. Potentiometric work on rosinduline 2G alone showed that its redox potential at this pH was −0·635 V against S.C.E. and using this value it was possible to calculate the percentage of oxidized rosinduline 2G at each potential throughout titration. These values were used to correct the $K_3Fe(CN)_6$ titres by adding appropriate volumes (see Table 4). The plot of electrode potential against $K_3Fe(CN)_6$ titre gave a curve of the correct sigmoid shape (Fig. 3) with an approximate $E_0'$ of −0·355 V. This constant, however, could be calculated more accurately by applying the Reed and Berkson (1929) method of analysis. To do this, it was necessary to estimate the amount of oxidized nicotinamide

present before addition of rosinduline 2G. It was found that at the commencement of titration, 3·05 ml of nicotinamide were present in the oxidized form and 11·56 ml in the reduced form. Using these values gave $E'_0 = -0\cdot360$ V against N.H.E. at 30 °C. The last column of Table 4 was then calculated by using the complete electrode equation

$$E = -0\cdot601 + 0\cdot03 \log \frac{3\cdot05 + y}{11\cdot56 - y}, \quad \ldots\ldots\ldots\ldots \quad (1)$$

where $y$ is the amount of oxidant added (ml). In Figure 3 the continuous line represents the results calculated from this analysis and the experimental points agree well with this theoretical curve for a 2-electron transfer.

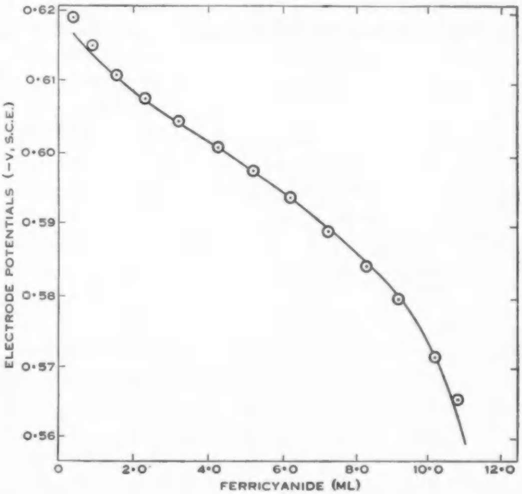



**Fig. 3.—Oxidative titration curve for methylnicotinamide. The points shown are experimental and the curve is fitted from equation (1) for a 2-electron transfer.**

The end-point of the titration (11·56 ml) suggested that only 57·8 per cent. of the original nicotinamide methiodide was reduced although the current passing during electrolysis suggested 80 per cent. reduction. This low value was partly accounted for by the presence of 3·05 ml oxidized amide which represented 15·2 per cent. of the original nicotinamide methiodide.

In other titration experiments electrolyses were conducted at pH's 5·15, 7·0, and 9·1 and at cathode potentials of −1·3 to −1·4 V and −1·7 to −1·8 V against S.C.E. Values of $E'_0$ at pH's below 9·1 were not obtainable due to rapid decomposition of dihydromethylnicotinamide during and after electrolysis. The results for pH 9·1 are summarized in Table 5 in which $E'_0$'s were calculated merely by determining the end-points of the titration curves and recording the

$E_h$'s at titres of a half this value. The table shows that the $E'_0$ of dihydromethylnicotinamide at pH 9·1 is independent of the cathode potential at which it was reduced and suggests that the ultimate product of electrolysis is the same whether reduction occurs by the mechanism of the first or second polarographic wave.

TABLE 5

OXIDATIVE TITRATIONS OF REDUCED METHYLNICOTINAMIDE WITH $2 \times 10^{-2}$M $K_3Fe(CN)_6$ AT pH 9·1 IN PRESENCE OF 5 PER CENT. ROSINDULINE 2G

| Electrolysis Conditions (pH 9·1) | | | Titration of 100 ml Reduced Compound | | |
|---|---|---|---|---|---|
| Cathode Potential (—V against S.C.E.) | Concentration ($M \times 10^{-3}$) | Current Efficiency (%) | End-Points (ml) | Reduced Compound (%) | $E'_0$ (—V) |
| 1·0/1·3 | 2 | — | 7·6 | 38 | 0·362 |
| 1·2/1·35 | 2 | 62 | 8·0 | 40 | 0·354 |
| <1·45 | 2 | 63 | 9·0 | 45 | 0·366 |
| 1·7/1·8 | 2 | 69 | 13·5 | 67 | 0·360 |
| 1·7/1·8 | 2 | 80 | 11·6 | 58 | 0·360 |
| 1·7/1·8 | 1 | 77 | 11·0 | 55 | 0·352 |
| 1·3/1·4 | 2 | 69 | 8·5 | 42 | 0·354 |

## IV. Discussion

An important observation during Karrer's work was the intermediate orange-yellow colour which was frequently noticed during reduction of nicotinamide methiodide with alkaline dithionite solution. Adler, Hellström, and v. Euler (1936) recorded the same phenomenon on reducing coenzyme I under similar conditions and attributed the transient colour to the unstable intermediate compound now regarded as a semiquinone-type free radical. The same stepwise reduction is probably responsible for the dimerization suspected during electrolytic reduction. It is possible to estimate the extent of dimerization during cathodic reduction when the results of potentiometric titration of the product are taken into account. For the experiment already described in detail, if $U$ is the percentage of methiodide remaining unreduced during electrolysis ; $O$ is the percentage which re-oxidized after electrolysis but before titration ; $R_i$ is the percentage which was reduced before any re-oxidation occurred ; $D$ is the percentage which dimerized during reduction, then $(U+O)$ is 15·2 per cent., $(R_i-O)$ is 57·8 per cent., $(U+R_i+D)$ is 100 per cent., and $(R_i+D/2)$ is 80 per cent. Solving these equations suggests that 6·5 per cent. of the original methiodide was unreduced during electrolysis and that 27 per cent. received a 1-electron transfer and subsequently dimerized. Of the 66·5 per cent. which was fully reduced to the dihydro-compound, 8·7 per cent. was re-oxidized before titration commenced and the remaining 57·8 per cent. was responsible for the titration curve.

From the behaviour of reduced methylnicotinamide towards dyes it appears that most reductions which would be expected purely on the grounds of $\Delta E_0$'s do in fact occur but that the rates are pH dependent. On the other hand, under the alkaline conditions used, the reduced compound oxidized extremely slowly in air, suggesting that rates of oxidation do not necessarily run parallel with the free-energy changes of the overall reactions.

The behaviour of the reduced compound towards excess ferricyanide suggests that after the first rapid reaction a weakly reducing component is attacked. Karrer has suggested that reduction of nicotinamide methiodide with dithionite may produce both *o*- and *p*-dihydro-substances. The latter compound is probably the weakly reducing component which is attacked slowly by excess ferricyanide and more rapidly by ceric sulphate. The same interpretation may explain the very slow secondary reduction of 2,6-dichlorophenolindophenol which was more noticeable with "aged" preparations of dihydromethylnicotinamide. The rapid loss in reducing power which the substance undergoes in acid solution and more slowly at pH 9 may be due to a rearrangement of the *p*- to the *o*-dihydro-form.

The effect of agitation on the electrode potentials of dihydromethylnicotinamide during oxidative titration has a parallel in the work of Michaelis and Eagle (1930) on oxazines. Stiehler, Chen, and Clark (1933) have suggested a tautomeric rearrangement of safranines during their reductive titration since they too show a similar behaviour. Due to the limited electrode agreement and the effect of mixing, the value of $E'_0$ which has been found for nicotinamide methiodide can be quoted to an accuracy of only $\pm 0 \cdot 02$ V. As the reduction involves a 2-electron transfer and assuming that the dissociation constant of the compound is outside the experimental pH range, the value of $\Delta E'_0/\Delta$ pH would be $0 \cdot 03$ V, and at pH $7 \cdot 1$ the $E'_0$ would therefore be $-0 \cdot 30 \pm 0 \cdot 02$ V against N.H.E. This value is to be compared with the $-0 \cdot 320$ V given by Burton (1952) for coenzyme I and the very approximate value of $-0 \cdot 40$ V which Karrer has quoted for the methyl compound.

## V. Acknowledgments

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# THE KINETICS OF OXIDATION OF *N*-METHYLACRIDAN: A MODEL FOR COENZYME I

By S. J. Leach,* J. H. Baxendale,† and The Late M. G. Evans‡

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*Summary*

The rates of oxidation of *N*-methylacridan by 2,6-dichlorophenolindophenol have been measured between pH 2·74 and 6·91 at 20 to 22 °C in the presence of 8 to 22 per cent. ethanol. The reaction was of the second order and was found to proceed by two simultaneous mechanisms, both involving the conjugate acid of *N*-methylacridan. The oxidation of this cation by the indophenol anion proceeded at a rate which was 19 times greater than the oxidation by the uncharged indophenol molecule. It is shown that oxidation probably occurs by hydrogen atom transfer rather than electron transfer. A similar mechanism for the oxidation of dihydro-coenzyme I would account for its slow reactivity towards molecular oxygen and the biological necessity for mediating systems involving both hydrogen atoms and electrons.

## I. Introduction

Coenzyme I (Burton 1952) and the model substance nicotinamide methochloride (Leach, Baxendale, and Evans 1953) are known to have extremely negative redox potentials but in spite of this, neither substance is as reactive towards molecular oxygen as many systems of higher $E'_0$. Apart from the general interest attaching to the mechanism of oxidation and reduction at nitrogen centres, it was considered that a study of the kinetics of oxidation of such systems might suggest a reason for their unusual specificity towards oxidants. The use of dihydromethylnicotinamide as a model is unsatisfactory owing to the presence of both the *o*- and *p*-dihydro-forms (Karrer *et al.* 1936; Leach, Baxendale, and Evans 1953) and the consequent difficulty in interpreting the kinetics. *N*-Methylacridinium chloride suggested itself as a more suitable model since it contains the quaternary ammonium nitrogen atom but can produce only the *p*-dihydro-compound on reduction.

The oxidation of this compound by 2,6-dichlorophenolindophenol has therefore been studied under a variety of pH conditions. The indophenol** dye was chosen as an oxidant since (*a*) its redox properties have been extensively

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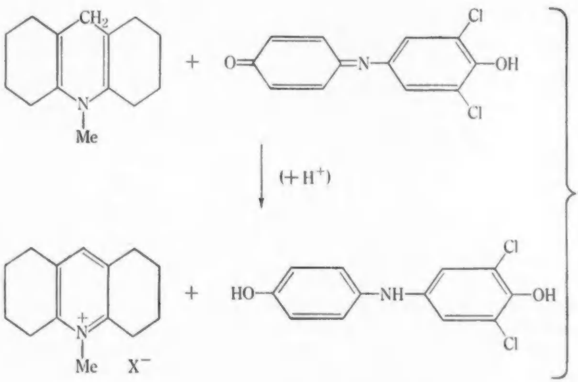
** Indophenol will be used in this paper to mean 2,6-dichlorophenolindophenol.

studied (Baxendale and Lewin 1946) and (*b*) the dye may be estimated absorptiometrically and the progress of its reduction to the colourless leuco-form can thus be conveniently and accurately followed.




.........(1)

## II. Experimental

(*a*) *Materials*.—(i) N-*Methylacridan*. Purified acridine was methylated with $Me_2SO_4$ (Kaufmann and Albertini 1909) and the product salted-out as *N*-methylacridinium chloride. This was reduced with sodium dithionite in buffer of pH 7, thoroughly washed, and then purified by recrystallization from ethanol-water. The properties of this reduction product and that obtained by electrolytic reduction at a cathode potential of —1·7 to —1·8 V agreed with those observed by Pictet and Patry (1902) and by Pope and Howard (1910). The product could be stored in the dark at 0 °C with little loss in reducing power. Ethanolic solutions of the product stored under these conditions decreased in purity from *c*. 97 to *c*. 91% (based on the reduction of indophenol) in 12 days.

(ii) *2,6-Dichlorophenolindophenol*. The purity of the commercial product (Hofmann La Roche) was increased from 80–95 to 96·5% by salting-out several times. A solution of the leuco-dye could be produced by reduction at pH 4·8 with platinized asbestos and $H_2$ until colourless. Usually, however, the reduction was effected in concentrated solution using sodium dithionite and the sparingly soluble product was collected and dissolved in ethanol for estimation. Its purity, based upon its reducing capacity, was usually greater than 93%.

(*b*) *Methods*.—(i) *Estimation of 2,6-Dichlorophenolindophenol*. Three methods were used: (1) Reduction with Pt-$H_2$ at alkaline pH's, followed by oxidative potentiometric titration with $K_3Fe(CN)_6$ in an atmosphere of $N_2$; (2) titration with standardized ascorbic acid using similar conditions to those of Bessey and King (1933); (3) comparison of the absorptions of test solutions with those of dye solutions of known strength. Calibration curves were constructed using indophenol samples which had been estimated by the first two methods. Absorptions were measured in acetate buffer of pH 4·7 using a Spekker absorptiometer with the appropriate colour filters and the solutions, which varied from $8\times10^{-6}$ to $9\times10^{-5}$M obeyed Beer's law throughout. The leuco-dye was estimated by adding a known quantity in ethanol solution to benzoquinone at pH 4·7, and measuring the absorption of the regenerated red colour.

(ii) *Estimation of* N-*Methylacridan*. A known amount of colourless *N*-methylacridan was added to a solution of 2,6-dichlorophenolindophenol in acetate buffer of pH 4·7, transferred to a 1 or 4 cm cuvette, and the absorption recorded (using a green filter) until it reached a constant value. This usually required 20–30 min and the final value was compared with that of a blank

solution of similar composition containing no reductant. The reducing-equivalent was then calculated by reference to calibration curves for indophenol constructed for pH 4·83 and 8·3% ethanol. In this way it was found that 1 mol of pure *N*-methylacridan reduced 1 mol of indophenol.

(iii) *Potentiometry*. Potentiometric titration of *N*-methylacridinium chloride in ethanolic (25%) glycine buffer with sodium dithionite in the presence of various potential mediators showed that the $E_0'$ was in the neighbourhood of —0·43 V against N.H.E. at pH 9. The potentiometric titration of various inorganic and organic redox systems, including 2,6-dichlorophenolindophenol, with an ethanolic solution of *N*-methylacridan confirmed the fact that the difference in redox potential between this substance and the indophenol dye was so great that the reaction went to completion.

(iv) *Kinetic Measurements*. At pH's below 6 the red indophenol was slowly reduced to the colourless form by *N*-methylacridan and at higher pH's the colour change was from deep blue to colourless. The methylacridinium salt produced in the reaction had a green fluorescence but showed the same absorption as pure water blanks. The reaction was therefore followed by an absorptiometric method using the appropriate colour filters and calibration curves for the two forms of the dye. The reactions were carried out at *c*. 20 °C.

Between pH 4·3 and 5·2, where the leuco-dye was not air-oxidizable, the conditions were similar to those described for the estimation of *N*-methylacridan. At lower pH's the reaction was extremely rapid and it was necessary to use lower concentrations of reactants. This meant the use of a specially constructed absorptiometer with a 13 cm light-path and a capacity of 600 ml. This instrument also carried provision for conducting the experiments in an atmosphere of $N_2$; it was therefore used also for experiments above pH 5·7 where the leuco-dye readily oxidizes in the air.

pH measurements were made with the glass electrode and it was observed that for phosphate, acetate, and citrate buffers there was a linear relationship between pH and the percentage of ethanol present. Calibration curves for the absorption of the indophenol at various concentrations were linear only at lower ethanol concentrations. It was also observed that, whilst the indophenol usually faded at pH's below 4·3, the colour was much more stable in the presence of 20% ethanol and measurements could be made satisfactorily even at pH 2·74.

Ionic strengths were not kept constant as it was found that these had a negligible effect upon either the colour of the dye or the rate of reduction.

## III. Results

### (*a*) *The Order of the Reaction*

It was found that when the initial concentrations of *N*-methylacridan $[Ac_R]$ and 2,6-dichlorophenolindophenol $[Ind_0]$ were of a similar order of magnitude, the plots of log $[Ind_0]/[Ac_R]$ were not linear. Figure 1 illustrates four typical cases. In these and other similar experiments it was possible to deduce consistent second-order rate constants, $k_2$, from the initial slopes of the curves. For the experiments of Figure 1 these were 1·43, 1·37, 1·23, and 1·30 l. mol$^{-1}$ min$^{-1}$ and the mean value for eight experiments was $(1{\cdot}29 \pm 0{\cdot}11) \times 10^4$ l.mol$^{-1}$ min$^{-1}$.

An investigation into the reason for the deviation from second-order behaviour showed that neither an increase in ethanol content to 21·4 per cent. nor the addition of reduced indophenol and/or oxidized acridinium salt affected the curvature. The latter is therefore due neither to the limited solubility of *N*-methylacridan nor to a back-reaction between the expected products. Furthermore, the possibility of an equilibrium may be discounted in view of the difference in $E_0'$ between the two systems. It was also shown that there was no

irreversible destruction of indophenol during the reaction, since the full colour intensity could be regenerated by the addition of benzoquinone. It was found, however, that the reaction was slowed down considerably by adding the actual products from a previous reaction. The reaction was therefore retarded by the formation of small amounts of a by-product of unknown structure. This product could not amount to more than a few per cent. of the total products in view of the stoichiometric observations already described. The side reaction probably affected mainly the acridine component since the departure from

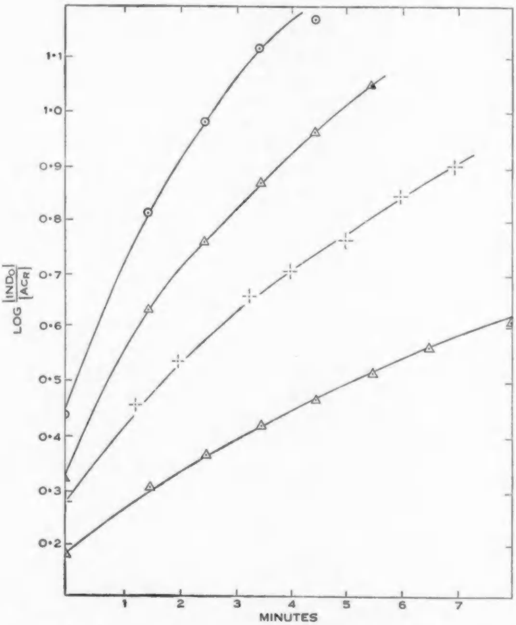



Fig. 1.—Second-order plots for the reaction of *N*-methylacridan with 2,6-dichlorophenolindophenol at pH 4·83.

second-order behaviour was increased as the proportion of *N*-methylacridan was decreased. This is borne out by the good agreement between the second-order constants obtained from initial slopes and those from pseudo-unimolecular experiments in which the *N*-methylacridan was in large excess. Three examples of the latter type are shown in Figure 2 and the first-order rate constants, $k_1$, are plotted against the concentration of *N*-methylacridan in Figure 3. The slope of the straight line obtained in this way gave a value of $1\cdot18\times10^4$ l. mol$^{-1}$ min$^{-1}$ for $k_2$. As will be seen later, it was found preferable to use values of $k_2$ derived from pseudo-unimolecular rate constants wherever possible, since these were more highly reproducible.

### (*b*) *The pH Dependence of Rate*

Rate measurements were carried out at six pH's between 2·7 and 6·9 at intervals of *c.* 0·8 and $k_2$ was computed as already described, both from the initial slopes of the second-order plots and from the slopes of the linear pseudo-unimolecular graphs. At pH's 6·16 and 6·91 the reactions were conducted in

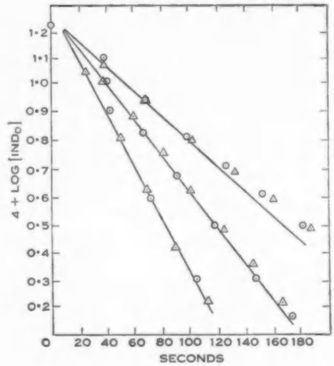



Fig. 2

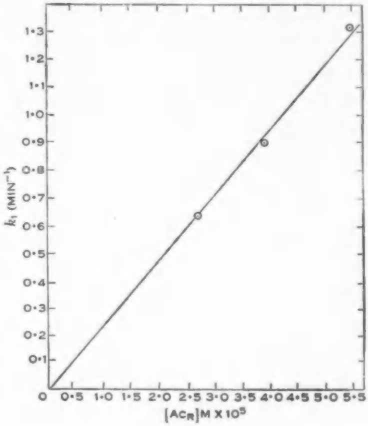



Fig. 3

Fig. 2.—Pseudo-unimolecular plots for the reaction of excess *N*-methylacridan with 2,6-dichlorophenolindophenol at pH 5·17.

Fig. 3.—First-order rate constants from the experiments of Figure 2, plotted against the concentration of *N*-methylacridan. Slope $=k_2=1 \cdot 18 \times 10^4$ l. mol$^{-1}$ min$^{-1}$.

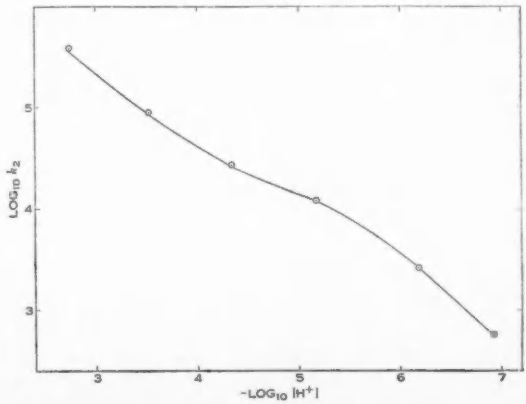



Fig. 4.—Log $k_2$ against log [H$^+$]. The points shown are experimental, whilst the continuous line is calculated using equation (**10**).

an atmosphere of $N_2$ and here values of $k_2$ were derived from the first-order rate constants only. It was found that the ionic strengths of the buffer media had a negligible effect upon the course of the reaction and this was borne out by the smooth curve obtained in the plot of log $k_2$ against log $[H^+]$ shown in Figure 4. The results obtained at all pH's are summarized in Table 1.

TABLE 1

CONDITIONS AND RATE CONSTANTS FOR THE OXIDATION OF *N*-METHYLACRIDAN BY 2,6-DICHLOROPHENOLINDOPHENOL

| Expt. No. | pH | Ethanol (%) | Initial [AcR] (mol $l.^{-1}$) $\times 10^6$ | Initial $[Ind_0]$ (mol $l.^{-1}$) $\times 10^6$ | $k_1$ ($min^{-1}$) | $k_2$ (mol $l.^{-1}$ $min^{-1}$) | $k_2$ Mean |
|---|---|---|---|---|---|---|---|
| 1 | 2·73 | 20·75 | 3·111 | 1·535 | — | $3·04 \times 10^5$ | |
| 2 | 2·76 | 20·75 | 3·111 | 1·770 | — | 3·64 ,, | $(3·8 \pm 0·5) \times 10^5$ |
| 3 | 2·73 | 20·75 | 2·157 | 1·729 | — | 4·51 ,, | |
| 4 | 2·74 | 21·1 | 5·366 | 1·790 | — | 4·08 ,, | |
| 4 | As above, but plotting in a first-order manner | | | | 2·030 | 3·78 ,, | |
| 5 | 2·74 | 21·1 | 7·740 | 1·745 | 2·719 | 3·51 ,, | |
| 6 | 3·52 | 20·75 | 3·111 | 1·78 | — | $9·69 \times 10^4$ | |
| 7 | 3·52 | 20·75 | 2·157 | 1·81 | — | 10·19 ,, | |
| 8 | 3·52 | 21·1 | 5·366 | 1·78 | — | 9·11 ,, | $(8·88 \pm 0·32) \times 10^4$ |
| 8 | As above, but plotting in a first-order manner | | | | 0·507 | 9·45 ,, | |
| 9 | 3·52 | 21·1 | 7·740 | 1·78 | 0·632 | 8·17 ,, | |
| 10 | 3·54 | 21·8 | 10·645 | 1·78 | 0·851 | 8·00 ,, | |
| 11 | 4·15 | 8·3 | 60·2 | 90·0 | — | $3·08 \times 10^4$ | |
| 12 | 4·15 | 8·3 | 38·1 | 90·0 | — | 3·45 ,, | |
| 13 | 4·15 | 8·3 | 27·8 | 90·0 | — | 3·22 ,, | |
| 14 | 4·34 | 21·2 | 13·85 | 14·35 | — | 2·72 ,, | |
| 15 | 4·34 | 21·2 | 10·11 | 14·35 | — | 3·29 ,, | |
| 16 | 4·34 | 21·2 | 109·45 | 14·35 | 2·415 | 2·21 ,, | |
| 17 | 4·34 | 21·2 | 69·25 | 14·35 | 1·870 | 2·70 ,, | $(2·73 \pm 0·18) \times 10^4$ |
| 18 | 4·34 | 21·2 | 54·54 | 14·35 | 1·513 | 2·77 ,, | |
| 19 | 4·35 | 21·8 | 15·36 | 3·51 | 0·439 | 2·86 ,, | |
| 20 | 4·96 | 8·3 | 77·3 | 89·6 | — | $0·62 \times 10^4$ | |
| 21 | 4·96 | 8·3 | 64·5 | 64·5 | — | 1·36 ,, | |
| 22 | 4·96 | 8·3 | 40·0 | 89·6 | — | 1·43 ,, | |
| 23 | 4·96 | 8·3 | 29·4 | 89·6 | — | 1·34 ,, | |
| 24 | 4·96 | 8·3 | 62·3 | 90·1 | — | 1·44 ,, | |
| 25 | 5·08 | 15·4 | 57·6 | 83·2 | — | 1·35 ,, | |
| 26 | 5·17 | 21·2 | 9·97 | 16·70 | — | 1·21 ,, | |
| 27 | 5·17 | 21·2 | 109·4 | 16·49 | 1·329 | 1·21 ,, | |
| 28 | 5·17 | 21·2 | 54·7 | 16·49 | 0·642 | 1·17 ,, | $(1·18 \pm 0·02) \times 10^4$ |
| 29 | 5·17 | 21·2 | 78·1 | 16·49 | 0·907 | 1·16 ,, | |
| 30 | 6·15 | 20·75 | 5·160 | 1·634 | $1·20 \times 10^{-2}$ | $2·33 \times 10^3$ | |
| 31 | 6·16 | 21·01 | 10·162 | 1·493 | 2·47 ,, | 2·43 ,, | |
| 32 | 6·17 | 21·27 | 14·942 | 1·146 | 3·86 ,, | 2·58 ,, | $(2·61 \pm 0·22) \times 10^3$ |
| 33 | 6·17 | 21·52 | 19·688 | 0·782 | 5·47 ,, | 2·78 ,, | |
| 34 | 6·18 | 21·77 | 24·482 | 0·482 | 7·47 ,, | 3·05 ,, | |
| 35 | 6·89 | 21·1 | 13·34 | 1·775 | $7·27 \times 10^{-3}$ | $5·45 \times 10^2$ | |
| 36 | 6·90 | 21·8 | 26·18 | 1·383 | $1·52 \times 10^{-2}$ | 5·82 ,, | $(5·67 \pm 0·15) \times 10^2$ |
| 37 | 6·92 | 22·4 | 38·96 | 1·030 | 2·25 ,, | 5·77 ,, | |
| 38 | 6·93 | 23·3 | 51·60 | 0·647 | 2·85 ,, | 5·52 ,, | |

The outstanding feature of these values is that the rate of reaction increases rapidly as $[H^+]$ is increased. Normally the rates of redox reactions increase as $[H^+]$ decreases, owing to the increasing concentrations of the anionic reactants. This exception to the general rule can be explained if we assume that the *N*-methylacridan reacts in a cationic form, that is, as its conjugate acid. When $[H^+]$ is high the formation of the cation is favoured whilst the formation of the indophenol anion is suppressed.

## IV. Reaction Mechanism

It has been shown that the rate of reaction is proportional to the total concentrations of *N*-methylacridan $[A_T]$ and 2,6-dichlorophenolindophenol $[I_T]$ respectively and also that the rate increases with increasing $[H^+]$.

Each of the reactants may exist in an ionic form, the *N*-methylacridan adding a proton to its tervalent *N* atom and the indophenol dissociating as an acid. That is

$$[AH^+] \rightleftharpoons [A]+[H^+] \quad \text{and} \quad [I] \rightleftharpoons [I^-]+[H^+]. \quad \ldots\ldots (2)$$

The acid dissociation constants for these equilibria are given by

$$K_A=[A][H^+]/[AH^+] \quad \text{and} \quad K_I=[I^-][H^+]/[I]. \quad \ldots (3)$$

Now

$$[A_T]=[AH^+]+[A] \quad \text{and} \quad [I_T]=[I]+[I^-]. \quad \ldots\ldots (4)$$

Combining (3) and (4)

$$[AH^+]=\frac{[A_T]}{1+K_A/[H^+]}, \quad \ldots\ldots (5)$$

$$[I^-]=\frac{[I_T]}{1+[H^+]/K_I}, \quad \ldots\ldots (6)$$

and

$$[A]=\frac{[A_T]}{1+[H^+]/K_A}, \quad \ldots\ldots (7)$$

$$[I]=\frac{[I_T]}{1+K_I/[H^+]}. \quad \ldots\ldots (8)$$

The possible reacting species are therefore A, $AH^+$, I, and $I^-$ and according to the pairs which react there will be four possible mechanisms. In the general case, when all four mechanisms are operative the rate of reaction will be given by

$$\text{rate}=k_t[AH^+][I^-]+k_t'[AH^+][I]+k_t''[A][I^-]+k_t'''[A][I], \quad \ldots (9)$$

and the concentration terms in this equation may then be expressed in terms of $[A_T]$, $[I_T]$, and $[H^+]$ by using equations (**5–8**). In order to account for the dependence of the rate constant $k_2$ upon $[H^+]$, it was first of all assumed that the rate was governed by only one of the terms in the full rate expression (**9**) and each was examined separately. In no case was there even an approximate fit to the experimental data and it may therefore be assumed that no single term in the full-rate equation is dominating. The possibility of two simultaneous mechanisms was therefore examined by taking the terms in (**9**) in pairs. It was found that of the six possible pairs of mechanisms all but one were again inconsistent with the experimental data for $k_2$ and $[H^+]$.

The remaining possibility fits the data precisely and this solution will be considered in some detail. For this case, the two operative mechanisms are

$$AH^+ + I^- \rightarrow \text{products} \quad \text{and} \quad [AH^+] + I \rightarrow \text{products},$$

and we may write

$$\text{rate} = k_t[AH^+][I^-] + k_t'[AH^+][I].$$

From (**5**), (**6**), and (**8**)

$$\text{rate} = \frac{k_t[A_T][I_T]}{(1+K_A/[H^+])(1+[H^+]/K_I)} + \frac{k_t'[A_T][I_T]}{(1+K_A/[H^+])(1+K_I/[H^+])},$$

and

$$k_2 = \frac{k_t}{(1+K_A/[H^+])(1+[H^+]/K_I)} + \frac{k_t'}{(1+K_A/[H^+])(1+K_I/[H^+])},$$

where $k_2$ is the observed rate contant and $k_t$ and $k_t'$ are true bimolecular rate constants which are independent of $[H^+]$. Or

$$k_2(1+K_I/[H^+]) = \frac{k_tK_I + k_t'[H^+]}{K_A + [H^+]}. \quad \ldots\ldots\ldots\ldots \quad (\mathbf{10})$$

For the first three groups of results in Table 1 (pH values 2·73, 3·52, and 4·34) since $K_I = 1{\cdot}4 \times 10^{-6}$, equation (**10**) simplifies to

$$k_2 = \frac{k_tK_I + k_t'[H^+]}{K_A + [H^+]}. \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (\mathbf{11})$$

By inserting in equation (**11**) the experimental values of $k_2$ and $[H^+]$ for the first three groups of results it is possible to evaluate the three unknown constants which were found to be:

$$K_A = 8{\cdot}41 \times 10^{-3} \text{ mol l.}^{-1},$$
$$k_t' = 2{\cdot}09 \times 10^{6} \text{ mol l.}^{-1} \text{ min}^{-1},$$
$$k_tK_I = 1{\cdot}33 \times 10^{2} \text{ mol}^2 \text{ l.}^{-2} \text{ min}^{-1}.$$

The value of $K_A$ found in this way means that for the last four groups of results in Table I (pH values 4·34, 5·17, 6·17, and 6·90), $K_A/[H^+] \gg 1$. Equation (**10**) then becomes

$$k_2(1+K_I/[H^+]) = K_I k_t/K_A + k_t'[H^+]/K_A. \quad \ldots\ldots \quad (\mathbf{12})$$

This equation may now be used to evaluate $K_I$. By inserting the values of $k_2$ and $[H^+]$ for the last four results it is found that $K_I = 3{\cdot}36 \times 10^{-6}$ g ions l.$^{-1}$. Substituting this value in the value already found for $k_t K_I$ we find that $k_t = 3{\cdot}96 \times 10^7$ mol l.$^{-1}$ min$^{-1}$.

The value found for $K_A$ is reasonable since Albert and Goldacre (1943) have shown that the $pK_A$ of the parent acridine (in the oxidized state) is 4·5 to 5·0 and reduction is known (Graebe and Caro 1871) to cause a marked decrease in basicity.

Clark and Cohen (1928) and Clark (1928) have given a value of $1{\cdot}58 \times 10^{-6}$ g ions l.$^{-1}$ for the dissociation constant ($K_I$) of 2,6-dichlorophenol-indophenol in $10^{-3}$ to $10^{-4}$M solutions but a value of $3{\cdot}16 \times 10^{-6}$ g ions l.$^{-1}$ for " more dilute " solutions. During the present study, the constant was determined from the kinetic analysis in 21·3 per cent. ethanol and at concentrations of between $10^{-4}$ and $10^{-6}$M and the value found was $(1{\cdot}35 \pm 0{\cdot}12) \times 10^{-6}$ g ions l.$^{-1}$. In more dilute solutions it is possible that the constant has a value nearer to that required by the above mechanism.

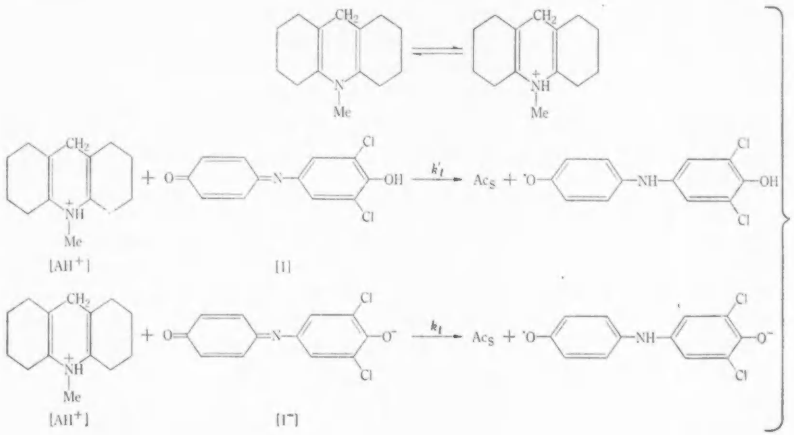
The smooth line in Figure 4 is drawn using the values of $k_2$ and $[H^+]$ calculated from equation (**10**) and the constants determined as above. It will be seen that the experimental points fit the curve closely and therefore that *N*-methylacridan is oxidized by way of its conjugate acid.

## V. Discussion

A large volume of work on redox systems has shown that a simultaneous two-equivalent transfer is extremely unlikely and it may be taken that the oxidation of *N*-methylacridan occurs in more than one step. There is weighty experimental evidence for the 2-step oxidation of the dihydroacridines. Thus, (1) in the final stage of synthesis of certain acridines the oxidation of the dihydro-compound invariably produces some dimer (Albert and Willis 1946), (2) the reduction of quinolines and pyridines at the dropping mercury electrode proceeds in two steps, and (3) during the reduction of *N*-methylacridinium chloride by sodium dithionite the solution passes through a dark green colour before it reaches the colourless fully reduced form.

The first oxidation step, which produces highly reactive free radicals, will be the slowest and will be responsible for the overall kinetics of the reaction. This step may proceed either by the loss of a hydrogen atom or the loss of an electron (followed by the addition of a proton to the indophenol). Since the acridan molecule is already positively charged in the reactive form, the electron mechanism may be ruled out: the energy requirements to produce a double positive charge on the molecule would be prohibitive. A hydrogen atom transfer step is therefore more probable, although whether this hydrogen is

detached from the nitrogen or the *p*-carbon atom cannot be decided. It seems most likely that the carbon atom is involved since the hydrogen in this position is more readily exchanged than that on the nitrogen atom (Brown and Letang 1941). The first step in the oxidation of the indophenol may therefore be written



. . . . . . . . . . . . . . . . . . . . (13)

in which $Ac_S$ represents the half-oxidized *N*-methylacridan free radical.

It has been shown that $k_t$ is greater than $k_t'$ by a factor of 19. This is to be expected on account of (1) the electrostatic attraction between two oppositely charged ions and (2) the greater symmetry and resonance stabilization of the indophenol free radical when the phenolic group is ionized.

The advantage of symmetry in the half-oxidized form may account also for the oxidation of *N*-methylacridan by way of its conjugate acid. This would have to be substantiated by detailed calculation of the net gain in resonance energy for each oxidation route. The problem is of general biological interest since many nitrogen compounds such as riboflavin (Kuhn and Ströbele 1937; Lingane and Davis 1941) and phenazines (Michaelis 1931, 1933; Preisler and Hempelmann 1937) exhibit highly coloured semiquinones only in acid solution. The work of Albert *et al.* (1945) on the antibacterial properties of acridines has also underlined the importance of the cation as the reactive species in certain biological systems.

The necessity for a hydrogen atom transfer mechanism may well account for the very slow reaction between *N*-methylacridan (and its structural analogues dihydromethylnicotinamide and dihydro-coenzyme I) with molecular oxygen. It has been observed both experimentally and theoretically by previous workers (Walsh 1948; Evans and Uri 1949) that oxygen is reduced preferentially by an electron transfer and in the absence of a mechanism which allows of electron

transfer, aerobic oxidation will therefore be a very slow process (compare the oxidation of hydrocarbons which requires very high temperatures).

These considerations may provide an explanation for the manner in which the various intermediates in the respiratory chain are " reached " by oxygen. In this sequence of oxidation-reduction reactions the aerobic oxidation of lactate, malate, triosephosphate, and other metabolites occurs via a number of intermediaries :

$$\text{substrate} \rightleftharpoons \underset{\text{enzymes}}{\text{pyridine nucleotide}} \rightleftharpoons \underset{\text{enzymes}}{\text{flavin}} \rightleftharpoons \text{cytochromes} \rightleftharpoons \text{oxygen}$$

the series being arranged in order of increasingly positive redox potential. In this manner, the oxidation energy of $O_2$ is released in a series of well-graded steps. Although such a series of systems presents a satisfactory thermodynamic picture, the necessity for the participation of each component has not yet been explained. One would expect, for example, that dihydro-coenzyme I ($E'_0$ $-0{\cdot}32$ V at pH 7) would react rapidly with oxygen ($E'_0$ $-0{\cdot}815$ V) whereas this reaction occurs very slowly if at all. In spite of the very favourable $\Delta G$ there appears to be some equally important factor preventing the interaction of these two systems. Our suggestion is that the two systems have no common redox mechanism, one reducing by electron transfer and the other by hydrogen atom transfer.

At the oxygen end of the process, electron transfer is more probable and the cytochrome systems, being $Fe^{++}$–$Fe^{+++}$ systems, are capable of reducing oxygen in this manner. At the other end of the chain, the substrate, which is non-ionizable at the site of reduction, may be oxidized only by the removal of a hydrogen atom and this is effected by coenzyme I. In this connection it is noteworthy that Fischer *et al.* (1952) have shown that lactic dehydrogenase catalyses a " direct stereochemically specific transfer of hydrogen atoms " between coenzyme I and the substrate without the formation of an intermediate which exchanges hydrogen with the medium. Between the two ends of the respiratory chain are the systems containing flavin-type coenzymes. Flavins contain both $>$CO and $>$NH groups and may therefore show the properties of quinones, which are known to reduce by electron transfer, and of nitrogen compounds of the type already discussed, which reduce by hydrogen transfer. On this view, the flavins might be regarded as potential mediators which are capable of receiving a hydrogen atom from coenzyme I and passing on an electron to the cytochrome system. The oxidation chain would then be " graded " not only with respect to the free-energy steps but also with respect to the chemical mechanism.

## VI. Acknowledgments

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# THE DEGRADATION OF TRISNORLANOSTENOLIC ACID BY THE CURTIUS REACTION

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*Summary*

As an alternative to the Barbier-Wieland degradation of trisnorlanostenolic acid (Curtis and Silberman 1952) degradation has been made through the Curtius reaction to the amine. This by quaternization and Hofmann deamination leads to the expected olefine which could serve as a starting point for further curtailment of the side chain.

## I. INTRODUCTION

In the degradation work of Voser *et al.* (1951) which led to the complete elucidation of the nature of the side chain of lanostadienol (Fig. 1), some modifications of the cyclic structure of the starting material were made to preserve it while the side chain was attacked by oxidizing agents. An alternative procedure for side-chain degradation which leaves the ring system unaffected, is reported here; it involves a Curtius reaction followed by Hofmann deamination.

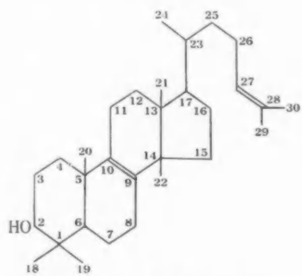

Fig. 1.—Lanostadienol.

The azide of the unacetylated trisnorlanostenolic acid gave a good yield of the urethane (70 per cent.) on decomposition in ethanol, but in acetic acid, gave a small yield (30–35 per cent.) of amine. The Curtius reaction with the azide of the acetylated trisnorlanostenolic acid proceeded more readily and the yields of amine were higher (60–65 per cent.). The Hofmann deamination of quaternary bases has been used by Julian, Mayer, and Printy (1948) to convert the $C_{20}$-amino-derivatives of some trisnorcholanic compounds into the corresponding olefines. They obtained good yields of these olefines using potassium

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hydroxide in ethylene glycol as deaminating agent. When the method was used here upon 2-acetoxytetrakisnorlanost-9(10)-en-26-yl trimethylammonium iodide there was little deamination and almost exclusive formation of 26-dimethylaminotetrakisnorlanost-9-en-2-ol. MacPhillamy and Scholz (1949) when using the Hofmann deamination to form certain unsaturated steroids, followed the method of Woodward and Doering (1945) in which concentrated aqueous alkali is used at high temperatures (220 °C). In experiments using 3α-12α-diacetoxy-, 12α-acetoxy-, and 3α-acetoxynorcholan-23-yl amine the yields of olefines were respectively of the order : 22, 37, and 15 per cent. This method gave comparable results with our materials. From the quaternary methiodide of tetrakisnor-26-dimethylamino-9-lanostene-2-ol deamination gave a 25 per cent. yield of olefine and 55–65 per cent. of tertiary amine. Since Hanhart and Ingold (1927) emphasize the beneficial effect to be expected from the use of the strongest bases as deaminating agents the deaminations were made in absence of carbon dioxide. The low yield of olefine (25 per cent.) is unexpected, for Hughes and Ingold (1941) gave a value of 72 per cent. olefine for the deamination of an *iso*amyl quaternary ammonium hydroxide.

During the Hofmann decomposition no appreciable dehydration occurred from the 2-hydroxy group, but two olefines and two isomeric tertiary bases of the expected composition were isolated, presumably because at the elevated temperature (220 °C) the strong alkali caused epimerization of the $C_2$-hydroxyl group.

## II. Experimental

All melting points were taken in hard glass capillaries and are uncorrected.

(*a*) *Trisnorlanostenolic Acid Hydrazide.*—Methyl trisnorlanostenolate, m.p. 150–151 °C (1·4 g) and hydrazine hydrate (100% ; 2·1 g) in absolute ethanol (12 ml) were heated on a water-bath (24 hr), cooled, diluted with water, and the precipitate collected and washed. Crystallized from ethanol it formed colourless needles, m.p. 208–211 °C (1·1 g), $[\alpha]_D^{22}$ +39·9° (*c*, 0·6 in ethanol) (Found : N, 6·6%. Calc. for $C_{27}H_{46}O_2N_2$ : N, 6·5%).

(*b*) *Tetrakisnorlanost-9(10)-en-2-ol-26-ethylurethane.*—To the above hydrazide (210 mg) dissolved in 95% acetic acid (6 ml) at 5 °C, a solution of sodium nitrite (120 mg) in water (0·6 ml) was added slowly with stirring. The cold solution was stirred for ½ hr, and for a further ½ hr at room temperature. Dilution with ice (2 vol.) precipitated the crystalline azide which was filtered and washed with ice-water, dissolved in ether, and dried over sodium sulphate at 0 °C. Ethanol (15 ml) was then added, the ether slowly distilled off, and the ethanol solution refluxed (4 hr). After concentrating under reduced pressure and cooling, the solution was filtered to remove insoluble by-products and diluted with water. The precipitate, dissolved in benzene (15 ml) and light petroleum (5 ml), was chromatographed on alkali-free alumina (4 g ; activity II). The fractions eluted with benzene and benzene : ether (9 : 1 ; 125 mg) after repeated recrystallization from ether-light petroleum and acetone gave microscopic needles, m.p. 153–154 °C. These were very soluble in ether and benzene, less soluble in light petroleum and methanol, $[\alpha]_D^{22}$ +54·8° (*c*, 0·2 in chloroform) (Found : C, 75·7 ; H, 10·6 ; N, 3·3%. Calc. for $C_{29}H_{49}O_3N$ : C, 75·8 ; H, 10·7 ; N, 3·1%).

(*c*) *26-Aminotetrakisnorlanost-9(10)-en-2-ol.*—The acyl azide was prepared from the hydrazide (400 mg) as described above and immediately added to glacial acetic acid (28 ml) which was diluted by the water in the precipitate (3 ml) and further by water added (1 ml) so that the resulting mixture contained approximately 12% of water. The temperature was raised slowly to 80–85 °C and then maintained for 2 hr. Above 60–65 °C gas was evolved freely and most of the solid dissolved. Dilution with water, followed by evaporation under reduced pressure, left

a viscous residue which was dissolved in water, cooled (0 °C) and made alkaline to phenolphthalein with 2N sodium carbonate, and exhaustively extracted with ether. Dry hydrogen chloride was passed through the dried ether solution until acid to wet Congo red paper, part of the ether evaporated off, and the amine hydrochloride precipitated (0·19 g). Crystallization from ethanol-acetone containing a trace of hydrogen chloride gave fine white crystals (135 mg), m.p. 315–316 °C, $[\alpha]_D^{22}$ +65·4° (*c*, 0·6 in ethanol) (Found : N, 3·1 ; Cl, 7·8%. Calc. for $C_{26}H_{46}ONCl$ : N, 3·3 ; Cl, 8·4%).

(*d*) *2-Acetyltrisnorlanostenolic Acid.*—Trisnorlanostenolic acid (550 mg), pyridine (5 ml), and acetic anhydride (1·4 ml) were allowed to react at room temperature (16 hr), then heated on a steam-bath (5 hr), cooled, decomposed with ice, and acidified. The product after crystallization from ether-light petroleum sintered slightly at 202 °C and melted at 207–209 °C, $[\alpha]_D^{22}$ +48·5° (*c*, 0·4 in chloroform). Yield 90% (Found : C, 75·9 ; H, 10·0%. Calc. for $C_{29}H_{46}O_4$ : C, 75·9 ; H, 10·1%).

(*e*) *2-Acetoxytetrakisnorlanost-9(10)-en-26-ethylurethane.*—2-Acetyltrisnorlanostenolic acid (305 mg), dry light petroleum (60–70 °C, 10 ml), and pure thionyl chloride (2 ml) were refluxed (4 hr) ; the suspended solid dissolved and the solution turned yellow. Removal of excess thionyl chloride and solvent under reduced pressure gave a crystalline acid chloride which was freed from volatile impurities by evaporating twice with light petroleum. To a solution of the acid chloride in dry acetone (20 ml) at 0 °C there was slowly added with stirring (2–6 °C) a solution of freshly activated sodium azide (160 mg) in water (0·6 ml). The stirring was continued at 5 °C for 40 min after which the material was precipitated by the addition of ice and water (30 ml). It was filtered, washed, dissolved in ether, and worked up as described for the azide of the 2-hydroxy-compound. The crude material (257 mg) when dissolved in benzene-light petroleum (1 : 2, 18 ml) and adsorbed on neutral alumina (6 g ; activity II) gave, by elution with light petroleum containing increasing amounts of benzene, a material (180 mg) which crystallized from ether-light petroleum in microcrystalline prisms, m.p. 139–140 °C, $[\alpha]_D^{22}$ +66·2° (*c*, 0·6 in chloroform) (Found : C, 73·9 ; H, 10·3 ; N, 3·1%. Calc. for $C_{31}H_{51}O_4N$ : C, 74·2 ; H, 10·3 ; N, 2·8%).

(*f*) *2-Acetoxy-26-aminotetrakisnorlanost-9(10)-ene.*—The wet acyl azide (1·2 ml $H_2O$) from acid (600 mg) in a mixture of glacial acetic acid (24 ml) and water (7–8 ml) was slowly heated to 84–88 °C and held at this temperature for 2½ hr. The fine suspension which separated at room temperature was filtered off, the solvent vacuum distilled, and the residue diluted with ice and made alkaline (0 °C) with 2N sodium carbonate. When the suspension was exhaustively extracted with chloroform the amine was obtained from the extract as long dendritic needles (450 mg). The primary amine was characterized as its picrate which was formed from a concentrated benzene solution and after recrystallization from the same solvent had m.p. 184–188 °C (decomp. 192 °C), $[\alpha]_D^{22}$ +74·6° (*c*, 0·2 in ethanol) (Found : C, 62·1 ; H, 7·7 ; N, 8·4%. Calc. for $C_{34}H_{50}O_9N_4$ : C, 62·0 ; H, 7·7 ; N, 8·5%).

(*g*) *2-Acetoxytetrakisnorlanost-9(10)-en-26-yl Trimethylammonium Iodide.*—The crude primary amine (450 mg) was methylated by heating on a steam-bath (12 hr) with acetic acid (90% ; 0·22 g), formic acid (90% ; 650 mg), and formalin (34% ; 450 mg). The product was cooled, diluted with ice-water, made alkaline, and extracted with chloroform. The oil (460 mg) recovered on evaporation of the solvent was dissolved in benzene (3 ml) and methyl iodide (0·37 ml) added. A crystalline precipitate formed and after standing (15 hr) the mixture was refluxed (1–2 hr), cooled, diluted with twice its volume of dry ether, and the crystalline precipitate filtered and washed with benzene-ether and ether. One crystallization from benzene-ethanol gave slightly cream coloured prismatic needles (350 mg), m.p. 282–284 °C (Found : C, 62·5 ; H, 9·1 ; N, 2·3%. Calc. for $C_{31}H_{54}O_2NI$ : C, 62·1 ; H, 9·1 ; N, 2·3%).

(*h*) *Deamination of the Quaternary Ammonium Iodide.*—(i) The quaternary compound (340 mg) suspended in ethylene glycol (3·5 ml) and potassium hydroxide solution (50% ; 1·7 g) was heated at 140–145 °C (3 hr), 150–155 °C (3 hr), and 165–170 °C (2 hr). Little trimethylamine was evolved. Dilution with water and extraction with ether and benzene yielded slightly yellow crystalline material (221 mg), from which the pure tertiary amine was obtained by crystallization from benzene, m.p. 184–185 °C. Yield 182 mg, 80% (Found : N, 3·6%. Calc. for

$C_{28}H_{49}ON$ : N, 3·4%). From the benzene mother liquors only 5–7% of neutral waxy product was isolated. Methylation of the tertiary amine gave a methiodide, m.p. 304 °C in almost quantitative yield.

(ii) The quaternary compound (491 mg) in methanol (90–95% ; 20 ml) was shaken (4–5 hr) at room temperature with freshly prepared carbonate and alkali-free silver oxide (410 mg). The solution of the quaternary base was filtered in a $CO_2$-free nitrogen atmosphere, the silver salts washed with warm methanol (90%), and the combined filtrates evaporated to 3–4 ml. These were added with stirring to a solution of potassium hydroxide (1–2 g) in water (1 ml) at 100 °C. The alkali was contained in a nickel crucible with a loose lid having openings for the stirrer and for the passage of nitrogen. After addition the temperature was raised to 215–225 °C and the heating continued (35–40 min). After addition of water and extraction with ether, the ether extract separated into neutral (82 mg, 27%) and basic fractions (222 mg, 63%). The yields of neutral (25–30%) and basic (57–65%) fractions remained substantially unaltered in experiments in which the rate of heating, excess of alkali, and type of vessel (glass, nickel) were altered.

The neutral fraction (150 mg) was chromatographed by dissolving in benzene-light petroleum (1 : 2, 15 ml) and adding to a neutral alumina column (5 g ; activity I–II). The first eluates contained material (40 mg) which crystallized in large transparent plates ; from later eluates with benzene-light petroleum (1 : 1) the material (84 mg) crystallized in white feathery needles. The less strongly adsorbed compound was purified by filtering through alumina (1·5 g ; activity I) the solvent being a mixture of benzene (2·5 ml) and light petroleum (12·5 ml) and recrystallizing the product (33 mg) twice from ether containing a little methanol, m.p. 163–164 °C (sintered slightly 159–160 °C) (Found : C, 84·5 ; H, 11·6%. Calc. for $C_{26}H_{42}O$ : C, 84·3 ; H, 11·4%).

The strongly absorbed fraction (75 mg) was purified by dissolving in benzene (5 ml) and light petroleum (10 ml) and chromatographed on alumina (2·2 g ; activity I) ; the substance (40 mg) eluted with benzene and benzene-ether (9 : 1) was twice recrystallized from ether, ether-light petroleum, and methanol when it formed stout needles, m.p. 155–156 °C, $[\alpha]_D^{22}$ +7·1° (*c*, 0·2 in chloroform) (Found : C, 84·2 ; H, 11·6%. Calc. for $C_{26}H_{42}O$ : C, 84·3 ; H, 11·4%).

Separation of the tertiary amines was achieved by dissolving the basic fraction (334 mg) in benzene-light petroleum (1 : 1 ; 25 ml) and chromatographing on alumina (10 g ; activity I–II). The benzene-light petroleum eluate (1 : 1) contained material (65 mg) which after charcoal treatment was crystallized from a small volume of chilled low-boiling light petroleum, m.p. 105–106 °C. It is very soluble in all common organic solvents, $[\alpha]_D^{22}$ +78·1° (*c*, 0·4 in chloroform) (Found : C, 80·7 ; H, 12·2%. Calc. for $C_{28}H_{49}ON$ : C, 80·9 ; H, 11·9%). The fraction eluted with benzene-ether contained a material (68 mg) which crystallized readily from benzene and was identical with the substance obtained by decomposition of the quaternary methiodide in ethylene glycol, m.p. 185–186 °C, $[\alpha]_D^{22}$+32° (*c*, 0·4 in chloroform) (Found : C, 80·6 ; H, 11·6 ; N, 3·6%. Calc. for $C_{28}H_{49}ON$ : C, 80·9 ; H, 11·9 ; N, 3·4%).

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# THE NON-RESISTANT COMPONENTS OF THE WOOD OF *EUCALYPTUS REGNANS* F. MUELL.

## I. WATER-SOLUBLE HEMICELLULOSE

By C. M. Stewart*

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*Summary*

A xylosan uronide material has been extracted, by means of water, from the residues remaining after exhaustive methanol extraction (at 150 °C) of the wood of *Eucalyptus regnans* F. Muell. The fractions isolated from the xylosan uronide material appear to be chemically similar, the observed fractionation depending on physical properties.

## I. Introduction

During the hydrolysis of wood in a mildly acid aqueous medium, portions of the non-resistant† hemicellulose, and of the lignin, are liberated and dissolve.

For lignin, only those units which have been sufficiently degraded become soluble: the rest of the liberated lignin, other than that which has been irreversibly polymerized, may be dissolved by the action of organic solvents, such as methanol or acetone, on the solid residue remaining from the aqueous acid treatment (Katzen and Othmer 1942; Ralph and Wardrop 1946).

However, if the wood is hydrolysed in a mildly acid methanol medium, the lignin is dissolved as it is liberated, except for that portion which is irreversibly polymerized, although the polysaccharides remain largely insoluble (Bland *et al.* 1947). Thus water extraction of the residue remaining from a mildly acid methanol treatment should ensure dissolution of that portion of the non-resistant hemicellulose which has been liberated during the hydrolytic treatment.

The isolation and preliminary examination of such a methanol-insoluble, water-soluble polysaccharide fraction of the wood of *Eucalyptus regnans* F. Muell. has been carried out.

## II. Discussion

In this series of papers the term " polyuronide hemicellulose " will be replaced by a more accurately descriptive term, " glycosan uronide hemicellulose ". The term polyuronide is confusing when applied to a glycosan uronide because it suggests that such a substance is a polymer composed of uronic acid base units; whereas a glycosan uronide may be regarded as a polymer

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† Wood components are designated as *non-resistant* or *resistant* according to the relative ease of solubility in various acidic or alkaline reaction media.

whose repeating unit is a polyglycose (or glycosan) residue linked to an *O*-methylhexuronic acid residue through its hemiacetal hydroxyl. It has been shown by O'Dwyer and other workers that the glycosan uronides of pored woods often contain a relatively resistant nucleus of xylosan uronide or, more precisely, of hexaxylose *O*-methylglucosiduronic acid.

The analytical results listed in Table 1 are typical of the various analyses of glycosan uronide hemicelluloses as carried out by such workers as O'Dwyer (1934, 1940), Sands and Nutter (1935), and Mitchell and Ritter (1940). The fractions contain very little ash, as would be expected from the method of extraction, and give strong naphthoresorcinol colour tests as well as positive reactions for hexuronic acid according to the specific colour reaction of Dische (1947). The water-soluble material consists essentially of xylose and hexuronic acid residues, to which are attached acetyl and methoxy groups. By the methods of analysis used in this work the xylose and hexuronic acid residues would appear to be present in the molar ratio of 6 : 1. This result is in agreement with results obtained by earlier workers using the same methods. However, it should be noted that a lower percentage of hexuronic acid anhydride (14·3) was obtained by the method of Whistler, Martin, and Harris (1940) using the residues of fractions 1 and 2 remaining after the original work had been completed. If this value is accepted the molar ratio would be more nearly 7 : 1. Thus the material under investigation is a xylosan uronide, containing six or possibly seven xylose residues per each hexuronic acid residue.

The xylosan uronide fractions have low ash contents, similar chemical compositions, high methoxyl contents, low lignin contents and are water-soluble after mild acid pretreatment. These properties are discussed below.

(*a*) *Low Ash Content.*—In the past, glycosan uronide hemicelluloses have been extracted after, or by, the use of rather drastic chemical reagents, usually of an inorganic nature. Hence glycosan uronide hemicelluloses have been notable for their rather high ash content which persists even after numerous purifications, for example, by dialysis, electrodialysis, or repeated reprecipitation from alcohols.

(*b*) *High Methoxyl Content.*—The high methoxyl content, which is about twice that previously observed for glycosan uronides from *E. regnans* (Mackney 1940), is probably due to methylation during the methanol extraction of the wood. Schwerin (unpublished data) has shown that a similar xylosan uronide contains about 2·5 per cent. of ester methoxyl but no glycosidic methoxyl.

(*c*) *Low Lignin Content.*—This would be expected if it is assumed that the xylosan uronide is liberated from a lignin–glycosan uronide hemicellulose complex and if it is assumed, further, that all the soluble liberated lignin had been dissolved by the methanol during the methanol extraction. A small amount of lignin may be liberated and degraded into water-soluble units during the hot-water extraction of the non-resistant xylosan uronide ; however, such water-soluble units would undoubtedly be soluble in the ethanol used for the precipitation of the xylosan uronide.

(*d*) *Extraction by Water.*—In experiment (B) only 79 per cent. of the material extracted by water was recovered but, because the analyses of the material obtained during experiment (A) and of the two fractions obtained during experiment (B) are very similar, and because only 72 per cent. of the material taken for fractionation in experiment (B) was recovered, it seems likely that all, or nearly all, of the material removed by water extraction consists of xylosan

TABLE 1

RESULTS OF THE WATER EXTRACTION AND ANALYSIS OF A POLYSACCHARIDE FRACTION OBTAINED FROM YOUNG E. REGNANS TREES

| Determination | Experiment (B)* | |
|---|---|---|
| (1) Loss in weight of sawdust on water extraction (%) .. | 4·7 | |
| (2) Xylosan uronide recovered from water extract (%) .. | 3·7 | |
| (3) Xylosan uronide recovered, % of material extracted .. | 79·3 | |
| | Fraction 1 | Fraction 2 |
| (4) Weight of fraction, % of total recovered fractionated material .. .. .. .. .. .. .. | 45 | 55 |
| (5) Total recovered fractionated material, % of xylosan uronide recovered in (2) .. .. .. .. .. | 72 | |
| *Analysis of Fractions* | | |
| (6) Ash, % of sample taken for analysis .. .. .. | 1·0 | 0·4 |
| (7) Hexuronic acid anhydride (%)† .. .. .. .. | 16·8 | 17·4 |
| (8) Xylosan (uncorrected for furfural from uronic acid) (%).. | 74·9 | 77·4 |
| (9) Methoxyl (%) .. .. .. .. .. .. .. | 6·9 | 5·7 |
| (10) Acetyl (%) .. .. .. .. .. .. .. .. | 6·3 | 5·4 |
| (11) Klason lignin (%) .. .. .. .. .. .. .. | Negligible | Negligible |
| (12) Xylose residues : hexuronic acid anhydride residue .. | 5·9 | 5·9 |
| (13) Methoxy groups : hexuronic acid anhydride residue .. | 2·3 | 1·9 |
| (14) Weight average molecular weight .. .. .. | 5000 | 3000 |

* In (1) and (2) the results are expressed on the basis of dry original sawdust ; in (7)–(11) the basis is the weight of dry ash-free sample taken for analysis.

† According to method of Dickson, Otterson, and Link (1930) as modified by Campbell, Hirst, and Young (1938) ; however, a value of 14·3% was obtained (single analysis on small residual specimens of fractions 1 and 2) by method of Whistler, Martin, and Harris (1940).

uronide. As a check on this assumption an approximate xylosan balance may be calculated. The difference between the xylosan contents of the methanol-cooked sawdust before and after water extraction is 3·6 per cent. (16·2 – 12·6) on an original wood basis (see Section III). On the same basis the amount of xylosan recovered in the xylosan uronide, if it is assumed that all the material extracted by water is xylosan uronide, is about 76 per cent. of 4·7 (see Table 1), that is, 3·6 per cent.

The methanol extraction is virtually a delignification procedure, and it is known that delignification followed by hot-water extraction causes the dissolution of a portion of the polysaccharide fraction of wood. For example, Mitchell and Ritter (1940) obtained holocellulose from sugar maple by alternate chlorination and extraction with a solution of monoethanolamine in 95 per cent. ethanol. On water extraction (1 hr at 100 °C) of this holocellulose and fractionation of the glycosan uronide hemicelluloses in the water extract, by means of ethanol and acetone, these authors obtained two fractions which were chemically very similar; however, the fractions contained about 25 per cent. of what was presumably, hexosan or hexose. Likewise Mackney (1940) has carried out a cold-water extraction of *E. regnans* holocellulose which had been extracted (2 hr at 100 °C) previously with dioxane, and obtained a glycosan uronide hemicellulose fraction which contained about 21 per cent. of a substance which was, presumably, hexose or hexosan.

Thus, because the water-soluble xylosan uronide contains little hexose or hexosan, it is probable that the non-resistant glycosan uronide hemicellulose *in situ* contains hexose residues which are readily split off under mild hydrolytic conditions. It may be noted that, although the water-soluble xylosan uronide was extracted from the methanol-extracted residues by means of hot water, the material is soluble in water at room temperature.

(*e*) *Similarity in Chemical Composition of the Fractions.*—This is apparent from the constant ratio of xylose residues to hexuronic acid residues. Hence it is probable that the observed fractionation of the xylosan uronide is based on physical rather than on chemical properties.

Assuming a formula of six xylose residues per methyl *O*-methylhexuronate residue, complete acetylation of the available hydroxy groups, in each xylosan uronide fraction, would give a product with about 40 per cent. acetyl content in a yield of about 170 per cent. Fractions 1 and 2 gave yields of 150 and 125 per cent., with acetyl contents of 37·8 and 34·4 per cent., respectively.

For fractions 1 and 2 the intrinsic viscosities in chloroform are in the ratio of 5 : 3 and, assuming that both samples were equally polydisperse and equally affected by the acetylation treatment, this can also be taken as the ratio of their molecular weights (because $a=1$ in the revised Staudinger equation). The absolute molecular weights were determined from the revised Staudinger equation, using $k=150$ (Sookne and Harris 1945). The values for fractions 1 and 2, in the unacetylated state, were approximately 5000 and 3000, respectively.

Therefore the observed molecular weight differences between the fractions may account for the observed fractionation in aqueous ethanol. As was expected, the material of higher molecular weight precipitated first on the addition of ethanol to the aqueous solution of the xylosan uronide material. The average D.P. of fractions 1 and 2 are 5 and 3, respectively, because the xylosan uronide repeating unit has a " molecular weight " of about 1000, on the basis of one uronic acid and six xylose residues.

## III. Experimental

(*a*) *Preparation of the Xylosan Uronide Fractions.*—The wood used was from a 14-year old stem of *E. regnans* obtained at Powelltown, Victoria, in late summer 1946. The bark was removed and the trunk reduced to sawdust which was given three 24 hr extractions with cold water, followed by three similar extractions with water at 55–60 °C. The methanol extractions were carried out on dry sawdust as described by Bland *et al.* (1947). Briefly the sawdust was subjected to one 4 hr and then to nine successive 16 hr extractions, fresh methanol being added after the 4 hr and after each 16 hr extraction.

In experiment (A), dry residual sawdust (250 g) was heated with water (3 l.) for 16 hr on the steam-bath. The filtered residue was washed with a little water to give 3·24 l. of filtrate and washings; the liquor was added to ethanol (98%; 24 l.) and the white flocculent precipitate allowed to settle. The supernatant liquid was removed, the precipitate washed with ethanol, and dried in a vacuum desiccator (yield *c.* 3·6% on the basis of the original wood). This precipitate contained xylose and hexuronic acid anhydride residues in the ratio of 5·9 : 1 (uronic acid anhydride and xylosan, approximately 17·6 and 78·1%, respectively).

In experiment (B), dry residual sawdust (513 g) was extracted with water (5·5 l.) for 75 min at 95 °C. The liquor was filtered, the residue weighed immediately, and then air dried and reweighed. The 4·24 l. of filtrate was added to ethanol (98%; 40 l.). After settling for 16 hr, the supernatant liquid was siphoned off, and the precipitate treated as in experiment (A), and finally dried to constant weight in a vacuum oven at 45 °C.

The whole of the dried material was redissolved in water (2 l.) and added to ethanol (98%; 3·5 l.), and, after settling overnight, the supernatant liquid was siphoned off and the solid material centrifuged out. This material was suspended in aqueous ethanol (500 ml) and recentrifuged. The material was then dried, ground, and redried to give fraction 1.

The liquors from both centrifugal separations were combined and ethanol (98%; 6·5 l.) added. After the precipitate had settled the supernatant liquid was siphoned off; the solid material was then separated to give fraction 2. The liquors from the above centrifugal separations, on the addition of more ethanol, did not yield any further precipitate.

The xylosan contents of the residue from the methanol cooks and of the residue from the hot-water extraction were 16·2 and 12·6%, respectively (on an original wood basis).

(*b*) *Trial Cold-Water Extractions.*—Several small samples (2 g) of methanol-extracted sawdust were extracted with hot and cold water, with the following results (material extracted, on original wood basis):

| | |
|---|---|
| cold water (20 °C) for 2 hr | 5·0% |
| cold water (20 °C) for 70 hr | 5·5% |
| hot water (98 °C) for 1 hr | 5·5%. |

Thus, although the xylosan uronide was extracted with hot water in (*a*) above, cold water would have been satisfactory, provided a longer extraction time was used.

(*c*) *Molecular Weight Determination of the Fractions.*—The xylosan uronide fractions were dried, weighed into flasks (50 ml), and dissolved in pyridine (80%; 10 ml). The flasks were then placed in an ice-bath and a mixture containing acetic anhydride (20 ml) and pyridine (10 ml) was added slowly to each, with frequent shaking. After standing for 1 hr at room temperature the flasks were placed for 3 hr in a water-bath at 50 °C. The bath temperature was raised for 1 hr to 80 °C, and then allowed to cool to 20 °C. After 3 days the contents of the flasks (which were clear solutions) were poured into ice water (400 ml), and the fine white precipitate was collected on a sintered glass funnel. The acetate was washed with distilled water (200 ml) and dried in the vacuum oven at 45 °C for 5 hr.

Samples acetylated by the method of Howlett (1944) did not give products soluble in pyridine. The acetyl content was determined according to Howlett (1944). Fractions 1 and 2 gave yields of 150 and 125%, with acetyl contents of 37·8 and 34·4%, respectively.

A sample (0·1 g) of each acetylated fraction was dissolved in chloroform (50 ml), and the viscosities of the solution and pure solvent were determined in an Ostwald viscometer at 25 °C.

From the graphs given by Millett and Stamm (1947), for the variation of specific viscosity with concentration, it was apparent that the values at such dilution can be taken as intrinsic viscosities. For fractions 1 and 2 the intrinsic viscosities were 0·234 and 0·140, respectively.

(*d*) *Analytical Methods.*—Ash : ignition at $575 \pm 25$ °C ; uronic acid anhydride : according to the method of Dickson, Otterson, and Link (1930), with minor modifications by Campbell, Hirst, and Young (1938) ; xylosan : according to Dorée (1950) with modifications by Mackney and Reynolds (1938) ; methoxyl : according to the method of Vieböck and Schwappach (1930) with minor modifications by Clark (1932) ; acetyl : according to Wise (1944) ; Klason lignin : according to Ritter, Seborg, and Mitchell (1932), with modifications by Cohen and Dadswell (1931), Cohen (1934, 1936), and Meade (unpublished data)—no alkaline pretreatment was given.

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# THE NON-RESISTANT COMPONENTS OF THE WOOD OF *EUCALYPTUS REGNANS* F. MUELL.*

## II. POLYSACCHARIDE CONSTITUENTS

By C. M. STEWART† and D. H. FOSTER†

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*Summary*

It is shown that the wood of *Eucalyptus regnans* F. Muell., on hydrolysis with N sulphuric acid, yields an aldobiuronic acid containing 4-*O*-methyl-D-glucuronic acid and D-xylose residues.

The composition of the non-resistant hemicellulose of *E. regnans* wood is compared with hemicelluloses obtained from various sources by other workers. The evidence suggests that the non-resistant hemicellulose *in situ* is composed, basically, of a poly-(hexaxylose 4-*O*-methyl-D-glucosiduronic acid) complex containing, in addition, acetyl groups and possibly galactose residues.

## I. INTRODUCTION

This investigation was designed, primarily, to identify the uronic acid component of the wood of *Eucalyptus regnans* F. Muell. : this component is associated chiefly with the non-resistant wood constituents and therefore may be liberated by comparatively mild hydrolytic treatments. The secondary aim of the work was to gain a preliminary knowledge of the non-uronic acid polysaccharide constituents of the non-resistant components of *E. regnans* wood.

Many workers have identified the hexuronic acid components of pectins, gums, and mucilages (Hirst 1942). Although a number of workers have investigated the hemicelluloses of woods, there has been a singular lack of success in identifying the uronic acid components of many wood hemicelluloses. O'Dwyer (1926), by the hydrolysis of the hemicellulose A obtained by alkali extraction from beech wood, has recovered the barium salt of an acid which gave derivatives similar to those of glucuronic acid. Yaramori and Tachi (1950) have extracted the woodmeal of *Ulmus davidiana* Planch. var. *japonica* Nakai with alkali and subjected the purified hemicellulose to acid hydrolysis to yield xylose, glucuronic acid, and xyloglucuronic acid.

O'Dwyer (1934, 1939) has obtained, from the wood of English oak, a hemicellulose fraction which on hydrolysis yielded an aldobiuronic acid containing one methoxy group per uronic acid residue. Also Sands and Gary (1933) have obtained an aldobiuronic acid from mesquite wood hemicellulose : they demonstrated that the aldobiuronic acid contained a methoxy group and, because the uronic acid could not be identified as its potassium acid saccharate, they suggested that the methoxy group was attached directly to the uronic acid residue.

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Previously Anderson and Otis (1930) had had the same difficulty in identifying the uronic acid component of mesquite gum: however, they succeeded in obtaining a small quantity of potassium acid saccharate; they thus demonstrated that the uronic acid component was glucuronic acid (since no mucic acid was formed during the oxidation) and, because of the presence of methanol in the mother liquors from the crystallization of the potassium acid saccharate, suggested that the methoxy group was interfering with the formation of the saccharic acid. In view of the above difficulties it was decided, for the investigation of the uronic acid component of *E. regnans* wood, to use one of the more recent alternative methods of identification and to use, as a starting point for the identification of the uronic acid component, the aldobiuronic acid which is readily liberated by boiling *E. regnans* wood with dilute sulphuric acid (Foster 1948).

Slightly different methods of identification have been used by White (1948) and by Smith (1951) to demonstrate that the acid hydrolysate from mesquite gum contains 4-*O*-methyl-D-glucuronic acid residues. Smith's method of conversion to the α- and β-forms of the amide of the partly methylated uronic acid was applied to the crude barium aldobiuronate obtained by the dilute acid hydrolysis of *E. regnans* wood. Subsequent to the completion of this work it was found that Jones and Wise (1952*a*) had already applied a similar procedure to aspen wood (*Populus tremuloides* Michx.); they showed that hydrolysis of extractive-free aspen sawdust yields L-rhamnose, L-arabinose, D-xylose, D-galactose, xylobiose, xylotriose, 4-*O*-methyl-D-glucuronic acid, and several oligosaccharides containing uronic acids.

## II. Discussion

The hydrolysate from the N sulphuric acid treatment of *E. regnans* shavings was given an "after-hydrolysis" to hydrolyse polysaccharides to simple sugars.

The analysis of the partially purified barium aldobiuronates is listed in Table 1 and is typical of those containing approximately one methoxy group per uronic acid residue. The glycosidic methoxyl content of the barium aldobiuronate was shown to be nil; hence the methoxy group is non-glycosidic and, obviously, non-ester in nature. In itself the isolation of the amides of methyl 4-*O*-methyl-D-glucosiduronic acid indicates that the aldobiuronic acid, and consequently the original wood, contains 4-*O*-methyl-D-glucuronic acid residues (as constituents of the non-resistant polysaccharides). Again, because glucose residues are characteristically linked through positions 1 and 4, and because position 4 is methylated, the 4-*O*-methyl-D-glucuronic acid residue is probably a terminal unit. Further work is necessary to elucidate the structure of the aldobiuronic acid.

Table 1 shows that the yield of furfural, from the barium aldobiuronates of *E. regnans* wood and of jute fibre, is much lower than that which may be calculated for the sum of the expected yields of furfural from the pentose and hexuronic acid residues. However, Sarkar, Mazumdar, and Pal (1952) have shown that the yield of furfural, obtained by distilling the aldobiuronic acid from jute hemicellulose with 12 per cent. hydrochloric acid, is approximately

equal to the yield that may be calculated using the assumption that the *O*-methylhexuronic acid does not yield any furfural or methoxyfurfural. They also showed that the phloroglucide precipitate, obtained during the determination of the furfural yield from jute hemicellulose, contains no methoxy groups. From this evidence, and the fact that no methanol was evolved with the furfural,

TABLE 1

ANALYSIS OF BARIUM ALDOBIURONATES FROM E. REGNANS WOOD AND FROM JUTE FIBRE

| Determination | Theoretical (%) | Jute* (%) | *E. regnans* (%) |
|---|---|---|---|
| Barium .. .. .. | 16·8 | 16·5 | 16·0 |
| Carbon dioxide .. .. | 10·8 | 9·5‡ | 9·5‡ |
| Furfural .. .. .. | 20·8† | 21·0 | 22·8 |
| Methoxyl .. .. .. | 7·6 | 7·0 | 6·0 |

* From the results of Sarkar, Mazumdar, and Pal (1952).

† Assuming that *O*-methylhexuronic acid gives no furfural; otherwise, the value is 31%.

‡ Determined according to the method of Whistler, Martin, and Harris (1940).

they concluded that *O*-methylhexuronic acid seems to be incapable of yielding furfural on distillation with 12 per cent. hydrochloric acid. The results from the analysis of the aldobiuronate of *E. regnans* wood support their contention. In addition, Jones and Wise (1952*b*) have investigated the aldobiuronic acid from the wood of *P. tremuloides*. It may be calculated, from their results, that the

$$\left[\begin{array}{cc} —[4\ \beta\text{-D-Xyl}\ 1]_n— & 4\ \beta\text{-D-Xyl}\ 1— \\ & 2\text{ or }3 \\ & | \\ & 1 \\ \text{D-GA}\ 1— & 4\ \beta\text{-D-Xyl} \end{array}\right]_x$$

where $n=5$ for Japanese elm wood (Yaramori and Tachi)
$=7$–$8$ for *Phormium tenax* (McIlroy)
$x=10$–$12$ for Japanese elm wood (Yaramori and Tachi).

Fig. 1.—Possible general basic structure of a glycosan uronide hemicellulose.

yield of furfural from the aldobiuronic acid is about 22 per cent. Therefore it may be concluded that no correction, for furfural from uronic acid, is necessary when analysing substances containing *O*-methylhexuronic acids.

The work of McIlroy (1949) and of Yaramori and Tachi (1950) has suggested that the structure of the xylosan glucosiduronic acid type of hemicellulose is probably given by Figure 1. The work of White (1948), Smith (1951), Jones and Wise (1952*a*) and the present work demonstrate that in some hemicelluloses

4-*O*-methyl-D-glucuronic acid residues are present, that is D-GA in Figure 1 is replaced by 4-*O*-methyl-D-GA.

The structure of the non-resistant xylosan uronide from *E. regnans* (Stewart 1953), in terms of the structural representation in Figure 1, is possibly given by $n=4$, $x=$say, 1–7, and where the uronic acid component is a methyl 4-*O*-methyl-D-glucuronate residue. This xylosan uronide contains 1–2 acetyl groups per glucuronic acid anhydride residue. In addition Mitchell and Ritter (1940) have shown that a non-resistant glycosan uronide hemicellulose extracted by hot water, from holocellulose prepared from sugar maple, contains about 9 per cent. of acetyl (i.e. 2–3 acetyl groups per uronic acid anhydride). Generally

TABLE 2

CALCULATED AND OBSERVED COMPOSITIONS OF WATER-SOLUBLE XYLOSAN URONIDE FROM E. REGNANS WOOD

| Determination | Repeating Unit | | Water-Soluble Xylosan Uronide from *E. regnans* Wood (% obs.) |
|---|---|---|---|
| | 5 Xylose Residues 1 Aldobiuronic Acid Residue 2 —$OCH_3$ Groups 1 —$OC{\cdot}CH_3$ Group (% calc.) | 7 Xylose Residues 1 Aldobiuronic Acid Residue 3 —$OCH_3$ Groups 2 —$OC{\cdot}CH_3$ Groups (% calc.) | |
| Xylosan* | 75·5 | 77·0 | 76 |
| Uronic acid anhydride | 16·8 | 12·9 | 17 (or 14) |
| Methoxyl | 5·9 | 6·8 | 6·3 |
| Acetyl | 4·1 | 6·3 | 5·9 |
| Molecular weight of repeating unit | 1049 | 1369 | † |

* Uncorrected for furfural from uronic acid.

† The material was divided into two fractions which had average molecular weights of 3000 and 5000; these values, presumably, representing multiples of the repeating unit.

glycosan uronide hemicellulose preparations have been isolated by means of extraction with alkaline solutions; it is probable that such treatment would remove many acetyl groups from the glycosan uronide hemicellulose. This point must not be overlooked in the study of wood hemicelluloses because acetyl groups, if present as orthoacetates in the original wood, may have an important bearing on the problem of lignin-polysaccharide and/or polysaccharide-polysaccharide bonding.

Table 2 sets out the observed and two calculated compositions for the xylosan uronide of *E. regnans* wood. The second calculated composition agrees fairly closely with the observed values (Stewart 1953); this could indicate that $n=6$ in Figure 1.

The foregoing discussion points to the necessity for accurate determinations for furfural, uronic acid anhydride, etc. and clearly shows how relatively small errors in estimation may alter the calculated structure of a hemicellulose.

In order to form a tentative picture of the non-resistant polysaccharides of pored woods (e.g. *E. regnans*), it is best to consider the isolated xylosan uronide hemicelluloses as consisting of polymeric chains of D-xylose residues with short side chains of single aldobiuronic acid residues occurring at intervals along the main chain. Jones and Wise (1952*b*) have shown that 2-*O*-(4-*O*-methyl-α-D-glucuronosyl)-α-D-xylose is present in the acid hydrolysate from *P. tremuloides*. Thus single units of 4-*O*-methyl-D-glucuronic acid residues may occur as side chains. The D-xylose residues in the chain are probably linked through positions 1 and 4, thus leaving positions 2 or 3 available for linkages to the short side chains of aldobiuronic acid or uronic acid residues. Acetyl groups also are attached to the polymer chain or to the side chains.

As the glycosan uronide hemicelluloses are always isolated after a hydrolytic treatment, the native glycosan uronide hemicelluloses (or glycosan uronide hemicelluloses *in situ*) are probably linked to other wood constituents. The acetyl and carboxyl groups are probably involved in such linkages. For example, Sarkar, Chatterjee, and Mazumdar (1947), and Sarkar *et al.* (1948) have obtained evidence of a bond between uronic acid and lignin in jute by showing that the acid value is doubled on delignification or alkali treatment, and Foster, Schwerin, and Cohen (1950) have shown that the uronic acid of *E. regnans* wood is hydrolysed much more slowly than would be expected if it were associated only with pentosans.

The water-soluble xylosan uronide isolated from *E. regnans* wood is probably the more resistant component of the native non-resistant glycosan uronide hemicellulose (cf. O'Dwyer 1934, 1939). In other words, the native non-resistant glycosan uronide hemicellulose contains other carbohydrate constituents.

The sugars remaining after the separation of the barium aldobiuronates were examined by paper chromatography because countercurrent distribution methods of separation were shown to be impracticable. The chromatograms indicated the presence of large amounts of xylose, smaller amounts of galactose, and traces of glucose and mannose. D-Xylose has been characterized ; galactose occurs to the extent of approximately 1 per cent. (Stewart, Amos, and Harvey 1953) ; glucose is derived, in part at least, from the wood cellulose ; and mannose occurs to the extent of about 1 per cent. (Foster, unpublished data). Moreover, other hydrolysates, when examined by chromatography with several solvents and using different spraying reagents, have given spots corresponding to those given by the above-mentioned sugars ; hence, in the present paper, it will be assumed that the spots do represent the sugars listed above.

The various wood fractions examined (holocelluloses, hemicelluloses, and extracts therefrom) gave on acid hydrolysis xylose and galactose, with no apparent glucose or mannose. Positive evidence of galactose only was obtained from the water extract of a dried methanol extract (140 hr at 150 °C) from sawdust. Galactose is apparently liberated more rapidly than xylose and both of these sugars are liberated much more rapidly than glucose or mannose. Therefore it may be concluded that xylose and galactose are derived from the non-resistant polysaccharides, but glucose and mannose are derived from the

more resistant wood components. Thus galactose is the only carbohydrate, other than D-xylose (and 4-*O*-methyl-D-glucuronic acid), which is likely to be present in the non-resistant glycosan uronide hemicellulose of *E. regnans* wood. All chromatograms showed a spot whose $R_F$ value was somewhat less than that of D-glucuronic acid. The experiments reported suggest that very mild hydrolytic agents may be useful in elucidating the structure of the non-resistant polysaccharides.

## III. Experimental

(*a*) *Wood Sample.*—The wood used was collected and treated as described by Stewart *et al.* (1951).

(*b*) *Preparation of the Crude Barium Aldobiuronate.*—About 5 kg of the extracted wood was heated in a glass-lined, steam-jacketed, open reaction vessel for 3·5 hr at 97 °C with sulphuric acid (5% ; 70 l.). The wood residue was then filtered off and washed until the washings reached pH 4. The filtrate and washings were given an " after-hydrolysis " for 1 hr at 100 °C—the concentration of sulphuric acid being about 4%. Barium carbonate was then added to bring the pH of the liquor to about 6.

After filtering off the precipitate of barium sulphate, the liquor (100 l.) was concentrated, at about 40 °C, in a vacuum evaporator (long-tube film type) to a volume of about 3 l. The concentrate was filtered and added to ethanol (98% ; 60 l.). The precipitate was reprecipitated several times by dissolution in water and the addition of ethanol. The partially purified barium aldobiuronate (Ba, 16 ; uronic acid anhydride, 38 ; $CH_3O$, 6%) was finally obtained in a yield of about 1·5% of the original wood. No glycosidic methoxyl was detected when the method of Hoffpauir and Reeves (1949) was applied to the barium aldobiuronate.

(*c*) *Preparation of Methyl (methyl 4-O-methyl-D-glucosid)uronate and the Amides of Methyl 4-O-Methyl-D-glucosiduronic Acid.*—The crude barium aldobiuronate (15 g) was mixed with methanolic hydrogen chloride (8% ; 120 ml) and refluxed overnight. The precipitated barium chloride was removed by centrifuging and the clarified liquor neutralized with silver carbonate. After centrifuging off the silver chloride, the liquor was evaporated *in vacuo* to dryness, extracted with acetone, recentrifuged and evaporated *in vacuo* to yield 9·9 g of syrup.

This syrup was distilled at 122 °C (bath temperature 155 °C), under a vacuum of 0·07 mm Hg, to yield 6·3 g of syrup $[n]_D^{20}$ 1·484, $[\alpha]_D^{20}$ +88·5 ° (*c*, 2·8 in methanol), $CH_3O$, 29·2%. The distillate was dissolved in ammoniacal methanol (5N ; 200 ml) and allowed to stand overnight at room temperature. The mixture was subjected to vacuum evaporation until the amides crystallized, m.p. 205–210 °C (2·1 g). The solid mass of amides was triturated with ethanol and the crude α-form of the amide crystallized. Recrystallization from ethanol gave the amide of methyl 4-*O*-methyl-α-D-glucosiduronic acid as thick plates, m.p. 232 °C, $[\alpha]_D^{20}$ +152 ° (*c*, 0·36 in water).

The amide of methyl 4-*O*-methyl-β-D-glucosiduronic acid was obtained from the mother liquors and it was recrystallized from methanol as needles, m.p. 228 °C, $[\alpha]_D^{20}$ —62 ° (*c*, 0·25 in water). The mixed α- and β-forms of the amide gave C, 43·37 ; H, 6·75 ; O, 42·8 ; N, 6·51 ; $CH_3O$, 27·46%. Calc. for $C_8H_{15}O_6N$ : C, 43·46 ; H, 6·84 ; O, 43·40 ; N, 6·33 ; $CH_3O$, 28·06%. The α- and β-forms of the amide gave X-ray powder diffraction diagrams whose lines were identical with those given by samples received through the courtesy of Dr. F. Smith.

(*d*) *D-Xylose as a Constituent of the Barium Aldobiuronate.*—The mother liquor, remaining from the crystallization of the β-form of the amide, was treated with 0·1 N barium hydroxide until the evolution of ammonia had ceased. The barium hydroxide was then neutralized (to pH 6) with sulphuric acid and the mixture was evaporated *in vacuo* to dryness. The residue was triturated with ethanol and the ethanol solution was filtered and evaporated to dryness. When this material was treated according to the method of Breddy and Jones (1945) the dimethyl acetal of dibenzylidene D-xylose was obtained as needles, m.p. 211 °C (not depressed on admixture

with a sample prepared from authentic D-xylose). However, the D-xylose obtained in this way represented only 5% of the mother liquor syrup. In addition the barium aldobiuronate yielded 22·8% of furfural when boiled with 12% hydrochloric acid.

(e) *Sugars Liberated during the Preparation of the Crude Barium Aldobiuronate.*—(i) *Determination of Partition Coefficients.* The partition coefficients of D-xylose, D-glucose, and D-galactose for the collidine : water system were determined by dissolving D-xylose (0·5 g), D-glucose (0·5 g), and D-galactose (1 g) in the solvent mixture (50 ml; 25 ml each of aqueous and non-aqueous layers). The mixtures were placed in 100 ml stoppered flasks and shaken for 60 hr, the flasks being immersed in a water-bath at 25 °C. Aliquots of the aqueous and non-aqueous layers were analysed by the Somogyi (1945) method for the determination of sugars. The partition coefficients for D-glucose, D-xylose, and D-galactose were approximately 15, 12, and 10 respectively.

(ii) *The Isolation of* D-*Xylose.* The liquors remaining from the precipitations of the crude barium aldobiuronate (see above), were concentrated in the vacuum evaporator to yield 61 g of crystalline D-xylose from the first crystallization, $[\alpha]_D^{20}$ +19·0° (*c*, 5·0 in water); identical X-ray powder diffraction diagrams with an authentic sample of D-xylose. The second crystallization yielded 84 g of crude D-xylose.

(iii) *Paper Chromatography.* The mother liquors, remaining from the crystallization of D-xylose, were chromatographed on paper (Whatman No. 1) using the pyridine : water-ethyl acetate (1 : 2 : 2) solvent as recommended by Jermyn and Isherwood (1949). After spraying with aniline-phthalic acid reagent, spots were obtained in identical positions to those given by authentic samples of D-xylose, D-galactose, D-glucose, and D-mannose; the relative order of abundance was in the order named, the substance corresponding to D-xylose being most abundant.

(f) *Examination of Various Wood Fractions.*—The following samples were examined: chlorine and chlorite holocelluloses from *E. regnans* woodmeal; chlorite holocellulose from *E. regnans* cambial zone; and hemicelluloses from (i) the cold-water extract of the residue from methanol-extracted *E. regnans* sawdust (140 hr at 150 °C), (ii) the hot-water extract (1 hr at 95 °C) from the chlorine holocellulose, (iii) the 4% sodium hydroxide extract from *E. regnans* sawdust. The wood used for the preparation of the above samples was obtained from young trees (14-yr old) of *E. regnans* from Toolangi, Victoria.

The samples (*c*. 0·5 g) were hydrolysed with N sulphuric acid at 95 °C for various times up to 7 hr. Barium carbonate was used to neutralize (to pH 6) the filtered hydrolysates; after filtering off the precipitated barium sulphate, the liquors were concentrated to a volume of about 0·2–0·4 ml. The concentrated sugar syrups were examined by paper partition chromatography using the collidine : water system. The unknowns were run against authentic samples of sugars and D-glucuronolactone. All the above sugar syrups contained substances with the same $R_F$ values as D-xylose and D-galactose, with no indication of D-glucose or D-mannose. A spot (probably due to 4-*O*-methyl-D-glucuronolactone), situated between those given by D-xylose and D-glucuronolactone, was obtained in many cases.

In addition the hot-water soluble material remaining when the methanol extract (140 hr at 150 °C) of *E. regnans* sawdust was evaporated to dryness gave positive evidence of D-galactose only (or a substance with the same $R_F$ value as D-galactose).

## IV. Acknowledgments

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## SHORT COMMUNICATIONS

### A RECORDING A.C. POLAROGRAPH*

By G. BUCHANAN† and R. L. WERNER†

A recording A.C. polarograph, which has been assembled from readily available components, is described. The D.C. voltage was drawn from the motor-driven slidewire of a recording D.C. instrument while the applied A.C. voltage was derived from the 50 cycle mains which was reduced to 4 V by means

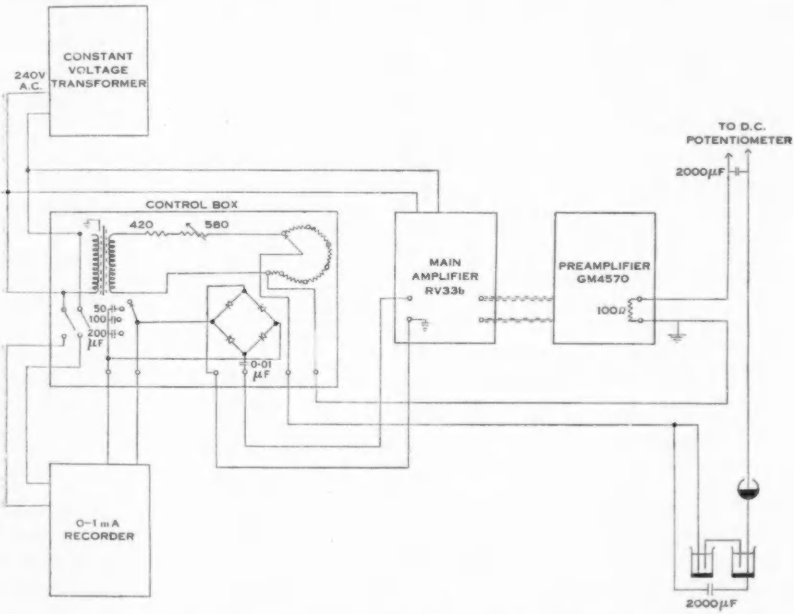


Fig. 1.—Schematic diagram of recording A.C. polarograph.

of a small transformer fitted with an electrostatic shield. This was then further reduced to millivolts by means of a variable resistance and a voltage divider consisting of six precision 1Ω resistors (Fig. 1).

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The combined D.C. and A.C. voltages were fed to the cell circuit through a 100Ω wire-wound resistance which was used to measure the A.C. by the voltage drop produced. The cell itself was quite conventional. The voltage appearing across the 100Ω resistance was initially amplified by a battery-operated single-stage preamplifier (Philips' type GM4570). The 100Ω load resistor in the cell circuit was wired across the input of the preamplifier inside the case to reduce stray pick-up. The output was further amplified by a vacuum tube voltmeter (V.T.V.M.) (Radiometer type RV33b). For manual work, the V.T.V.M. was read directly, but for recording, the amplified A.C. output was switched through a copper oxide rectifier bridge to an Evershed and Vignoles 1 mA recorder. A series of condensers (50, 100, 200 μF electrolytic) across the recorder allowed variations of response time so that records could be damped as necessary.

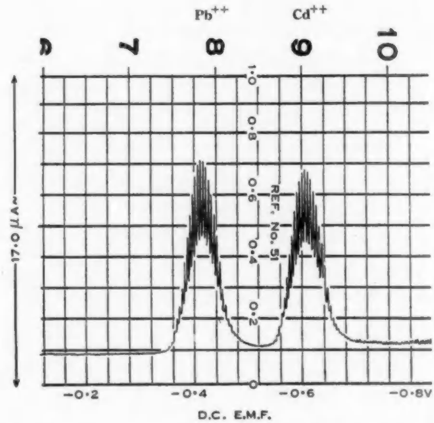


Fig. 2.—A.C. polarogram of $1 \cdot 0 \times 10^{-3}$M $Pb^{++}$ and $1 \cdot 0 \times 10^{-3}$ $Cd^{++}$ at 25 °C. Supporting electrolyte: 1·0M $HNO_3$+dissolved air; $m$, 2·70 mg $sec^{-1}$; $t$, 3·5 sec at zero applied potential. Sweep voltage 5·0 mV. A "pool condenser" saturated calomel electrode was used as a reference half-cell. The recorder was damped with a 100 μF electrolytic condenser.

In order to keep the series resistance of the cell circuit low (Breyer, Gutmann, and Hacobian 1950*a*) the calomel cell was by-passed with a large condenser (Breyer, Gutmann, and Hacobian 1950*b*) and also the potentiometer slidewire. A 2000 μF electrolytic condenser across the calomel cell was found to give curves whose shapes were identical with those produced by a mercury pool anode.

It was found convenient to adjust the impressed A.C. voltage in steps of 5 or 10 mV across each 1Ω resistance. This allowed ranges of 5–30 or 10–60 mV. The voltage was adjusted by switching the V.T.V.M. across the output of the voltage divider and adjusting the variable resistance.

A complete A.C. polarogram from 0 to −2·0 V can be recorded in 15 min with each drop clearly visible on the chart (Fig. 2). This speed of recording

reproduces the polarograms without distortion as judged by a comparison with manual plots.

Tests have shown that the whole system is quite linear with respect to current and that at maximum gain a current of $1 \cdot 7$ $\mu$A r.m.s. gave full-scale deflection on the recorder.

From Figure 2, it can be seen that $1 \cdot 0 \times 10^{-3}$M $Pb^{++}$ in $1 \cdot 0$M $HNO_3$, gave, with a sweep voltage of 5 mV r.m.s., an A.C. peak of $8 \cdot 5$ $\mu$A. That is, $1 \cdot 0 \times 10^{-4}$M $Pb^{++}$ would give $0 \cdot 85$ $\mu$A or half-scale deflection at $5 \cdot 0$ mV sweep and maximum gain. Since the peak height is proportional to the applied A.C. voltage, more dilute solutions may be conveniently dealt with by increasing the sweep voltage, providing this does not interfere with the resolution of two peaks whose $E_{\frac{1}{2}}$ are close.

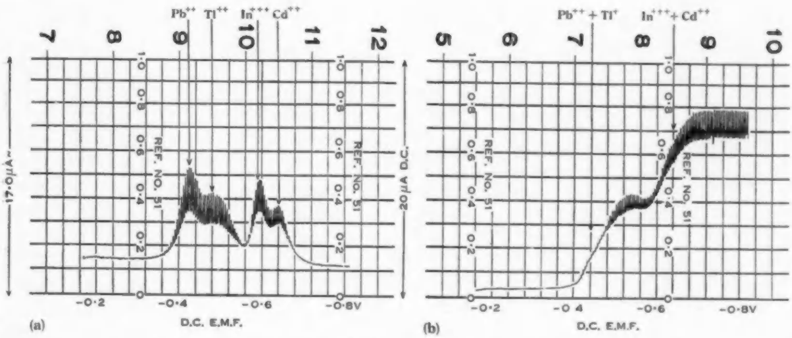


Fig. 3. (*a*).—A.C. polarogram of $2 \cdot 5 \times 10^{-4}$M $Pb^{++}$, $7 \cdot 7 \times 10^{-4}$M $Tl^{+}$, $5 \cdot 0 \times 10^{-4}$M $In^{+++}$ and $3 \cdot 75 \times 10^{-4}$M $Cd^{++}$ at 25 °C. Supporting electrolyte: $0 \cdot 5$N HCl (air free); *m*, $2 \cdot 70$ mg sec$^{-1}$; *t*, $3 \cdot 5$ sec at zero applied potential. Sweep voltage $5 \cdot 0$ mV. A " pool condenser " saturated calomel electrode was used as a reference half-cell. The recorder was damped with a 100 $\mu$F electrolytic condenser.

Fig. 3 (*b*).—The corresponding D.C. polarogram. The drop characteristics are the same as in Figure 3 (*a*). A saturated calomel cell was used as a reference half-cell. The recorder was damped with a 100 $\mu$F electrolytic condenser.

By plotting the output current against the input scanning voltage, with a fixed resistance in the circuit approximating to the ohmic resistance of the cell, it was found that the 50 cycle voltage picked up in the leads etc. was about $0 \cdot 5$ mV. Since normal scanning voltages of 15 mV were used, and since the pick-up was approximately constant, no interference resulted from this source. The unit has been in operation for some months and has proved completely reliable. Repeat polarograms taken over periods of some hours are reproducible to $0 \cdot 5$ per cent. or better.

This instrument has already been found to have considerable advantages over the corresponding D.C. instrument in the detection of metal ions in solution (Breyer, Gutmann, and Hacobian 1951). From Figures 3 (*a*) and 3 (*b*), it will be seen that four metal ions can be easily identified by their summit potentials

on the A.C. polarogram while the D.C. polarogram shows only two steps on the same solution. Work is continuing on the application of A.C. polarographic techniques to the quantitative determination of ions in mixtures and to physico-chemical problems.

The authors wish to thank Dr. F. Gutmann, N.S.W. University of Technology, for his helpful advice.

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# THE LANTHANON CONTENT OF KING ISLAND SCHEELITE ORE*

By R. C. Vickery†

The occurrence of lanthanons and their distribution in Swaziland scheelite was examined by Servigne (1940) and by Marsh (1943). Although these workers disagreed on the europium content of the ore they studied, the total lanthanon content observed (0·05 per cent. $Ln_2O_3$) was significant enough to warrant an examination of Australian scheelite for similar occurrences of lanthanons.

The initial scheelite investigated was from King Island and three samples were examined: (*a*) "run of mine" ore containing 0·6 per cent. $CaWO_4$, 10–20 per cent. $CaCO_3$, the balance being substantially andradite; (*b*) a primary concentrate from the first flotation beneficiation of the "run of mine" ore containing 12–19 per cent. $CaWO_4$, the remainder being $CaCO_3$; (*c*) the final "sales" material containing 83 per cent. $CaWO_4$, balance $CaCO_3$. Lanthanons were found only in the original ore, sample (*a*), to the extent of 0·4 per cent. The oxide isolated contained a radioactive constituent, 20 ml of solution containing 0·17 g oxide gave 33 counts/min ($\beta+\gamma$-radiation) above background. No radiation was observed from samples (*b*) or (*c*). These results suggested concentration of lanthanons and radioactivity in the primary flotation residues, and analysis of these residues gave 0·7 per cent. $Ln_2O_3$, and 0·2 g of oxide in 20 ml of solution gave 35 c/min above background.

Several accounts have been given of the geology and mineralogy of the King Island scheelite ore body and the most comprehensive mineragraphic report (Stillwell 1942) records the presence of more than 30 mineral species. The presence in the ore body of monazite or other lanthanon minerals was not reported and of the minerals observed only five have previously been reported as containing lanthanons: apatite, sphene, epidote, zoisite, and andradite. Garnets frequently contain lanthanons (Rankama and Sahama 1949; Jaffe 1951) but in such occurrences yttrium and the heavy lanthanons generally preponderate on account of their more suitable ionic size. Moreover, the occurrence of lanthanons is more to be expected in the spessartite garnets. Visual examination of the absorption spectrum of their solution showed the oxides derived from the flotation residues to be mainly light lanthanons and analysis of handpicked specimens of andradite showed no lanthanons therein.

Centrifugal separation of the flotation residue in methylene iodide (D : 3·325) yielded an apatite concentrate (float) in which no lanthanons could be detected. Nitric acid treatment of the flotation residues, which could be expected to decompose and dissolve much of the apatite, likewise, showed no lanthanon extraction. The residual minerals, epidote and sphene, together with much andradite, were subjected to magnetic and electrostatic separations

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which yielded 60 per cent. concentrates of each of these minerals. Of the lanthanons originally present 80 per cent. was found in the epidote concentrate. Although allanite (orthite) is a common lanthanon epidote neither detection of its presence, nor determination of the mode of occurrence of the lanthanons in the epidote was directly possible. Solutions of the epidote concentrate were radioactive but less so than a solution of New South Wales monazite containing the same amount of lanthanon oxides. Percentage distribution of extracted lanthanons in terms of the oxides was:

Ce 39·2; Pr 13·1; Nd 26·1; Sm 4·8; Eu 0·85.

No indications of the presence of dysprosium or erbium were found, and the balance of the oxides was probably lanthana since basicity separation in fused ammonium nitrate (Vickery 1949) yielded a lanthanum concentrate equivalent to 14·2 per cent. $La_2O_3$. Due to background absorption, the spectrophotometrically determined value for europium is likely to be some 5 per cent. too high. The ratios of elements generally are similar to those expected for allanite or monazite (Rankama and Sahama loc. cit.) although the Nd : Pr ratio is somewhat lower than usual and even deducting 5 per cent. from the europium value the Eu : Sm ratio is much higher than normally encountered in allanite or monazite. Such an increase in the Eu : Sm ratio was also observed by Marsh (loc. cit.) working on the Swaziland ore.

### *Experimental*

Of each of the original samples (*a*), (*b*), and (*c*) 200 g was decomposed in aliquots by fusion with sodium peroxide. The cooled mass was extracted with water and the residue washed free from alkali before dissolution in 50% $HNO_3$. Several alternate precipitations as hydroxide and oxalate were necessary for adequate removal of calcium and iron. In the presence of much iron, precipitation of lanthanon oxalates is always somewhat inhibited, and in some cases standing for a week was necessary to obtain precipitation of the lanthanon oxalates from the highly ferruginous solution.

Radioactivity was measured through an A.E.L. type SC100–1 instrument with counting periods of 3 hr or longer.

The methylene iodide separations were effected by centrifuging at 2500 r.p.m. in water-buffered tubes.

Spectrophotometric analysis of the final oxides was carried out on the Beckmann DU instrument utilizing the absorption wavelengths and extinction coefficients given by Moeller and Brantley (1950) the appropriate corrections being applied for interfering ions.

The author's thanks are due to Mr. E. S. Pilkington for carrying out check analyses of the ore samples, and to Dr. R. Segnit and Mr. A. J. Gaskin for helpful advice on minerals separation and for carrying out the magnetic and electrostatic separations.

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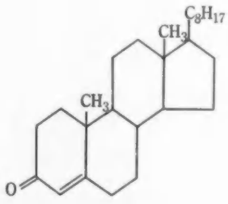
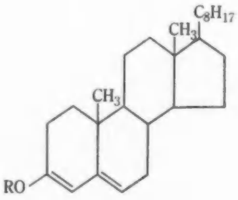
# A CONVERSION OF CHOLEST-4-EN-3-ONE INTO CHOLEST-5-EN-ONE*

By A. J. Birch,† Patricia Hextall,† and J. A. K. Quartey‡

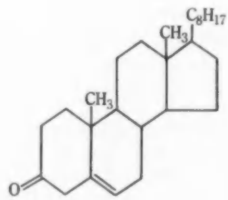
Cholest-4-en-3-one (I) has been converted into cholest-5-en-3-one (III) (Birch 1950) through the salt II (R=K) obtained by the action of potassium amide in liquid ammonia on the enol-acetate (II, R=Ac) of I. This type of movement of a double bond from a conjugated to an unconjugated position is potentially of considerable use in synthesis and has been employed in the total synthesis of some non-aromatic steroids (Cardwell *et al.* 1951, 1953 ; Woodward *et al.* 1951). In connection with other work in this field (Birch, Quartey, and Smith 1952) processes are being sought which are more practicable than that already used. The cholestenone conversion has been used as a model, although in the meantime the chief problem with which it is associated—the preparation in good yield of cholesterol—has been solved by a one-stage hydrolysis and reduction with sodium borohydride (Belleau and Gallagher 1951 ; Dauben and Eastham 1951). It is also of considerable interest that *tert.*-butyl magnesium chloride causes enolization of I to give II (R=MgCl) (Belleau and Gallagher 1951), although potassium amide failed to do so (Birch 1950). In view of these results we have abandoned further work on the preparation of cholesterol.



(I)



(II)



(III)

Potassium amide in ammonia was originally used merely to demonstrate the feasibility of the process ; it was realized that the reagent has drawbacks from a practical point of view. An attempt was first made to replace it with ethyl magnesium bromide. From the product cholest-5-en-3-one could be isolated in poor yield but was accompanied by a large proportion of non-ketonic material which was not further examined. Attention was then turned to methyl-

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anilinomagnesium bromide, which would be expected to cause fission of the enol-acetate by analogy with the reaction of anilinomagnesium bromide with esters (Hardy 1936). In fact, a 50 per cent. yield of III was obtained without difficulty. The reagent should be useful with molecules containing other active groups, and other ketones are now being examined. Attempts to prepare enamines by the action of piperidine in boiling benzene on I or II (R = $COCH_3$) did not give the compounds of the desired structure, since the action of acid regenerated I together with what may be polymers. According to the results of Mannich and Kniss (1941) any 3-piperidinocholesta-3,5-diene should have given rise to III.*

The Grignard reagent from magnesium (0·295 g; 3 mol), ethyl bromide and methylaniline (2·16 g; excess) in ether (25 c.c.) was cooled in ice and the enol-acetate (II, R = Ac) (1·70 g) in a small volume of ether added. After 1 hr at room temperature hydrochloric acid (5 per cent.) was added, the ether layer separated and rapidly washed with dilute acetic acid (10 per cent.), water, saturated sodium bicarbonate solution, water, and then dried (sodium sulphate). After evaporation of the solvent the residue was twice crystallized from ethanol to give cholest-5-en-3-one (0·75 g), m.p. 122–124 °C, $[\alpha]_D$ −2·2° (in chloroform). It was further characterized by reduction with lithium aluminium hydride to cholesterol, m.p. 144–145 °C, undepressed by an authentic specimen, m.p. 147–148 °C, $[\alpha]_D$ −39·5° (in chloroform).

This work was partly carried out during the tenure of the Smithson Fellowship of the Royal Society (A.J.B.) and of a maintenance grant from the Government of the Gold Coast (J.A.K.Q.). The authors are grateful to the Nuffield Foundation for financial assistance.

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* *Note added in Proof.*—Since this work was completed Heyl and Herr (1953) have shown that a 3,5-dienamine can be prepared using pyrrolidine. We confirm this, but find the substance unexpectedly difficult to hydrolyse, and under conditions leading to hydrolysis only the 4-unsaturated ketone is obtained.

# SOME OESTROGENIC 4-PHENYL-SUBSTITUTED *ISO*FLAV-3-ENS*

By R. B. Bradbury†

Since certain 2- and 4-alkyl-substituted *iso*flav-3-ens have been shown to be oestrogenic (Bradbury and White 1953), it was considered of interest to prepare 4-phenyl-substituted *iso*flav-3-ens, which are structurally analogous

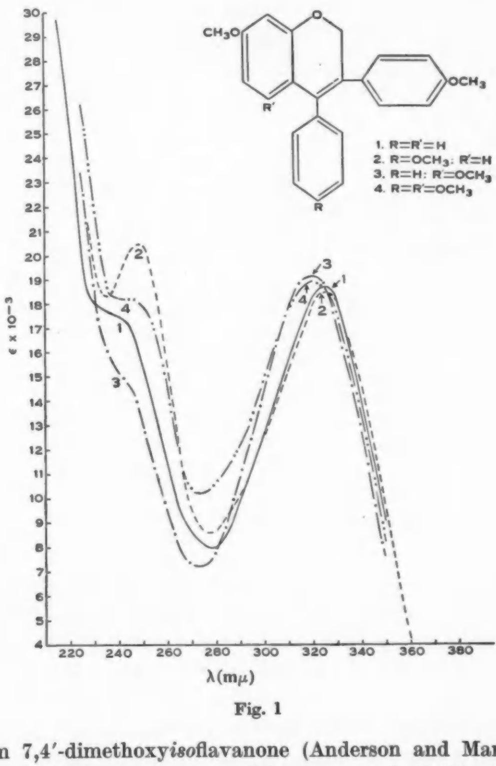


Fig. 1

to the oestrogenic triphenylethylenes (Schonberg *et al.* 1940). 7,4′-Dimethoxy-4-phenyl*iso*flav-3-en and 7,4′-dimethoxy-4-(*p*-methoxyphenyl)*iso*flav-3-en were prepared from 7,4′-dimethoxy*iso*flavanone (Anderson and Marrian 1939) and

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the corresponding Grignard reagent, while 5,7,4′-trimethoxy-4-phenyl*iso*flav-3-en and 5,7,4′-trimethoxy-4-(*p*-methoxyphenyl)*iso*flav-3-en were prepared from 5,7,4′-trimethoxy*iso*flavanone (Bradbury and White loc. cit.) in a similar manner.

The ultraviolet spectra of these compounds (Fig. 1) show that a *p*-methoxyl group on the 4-phenyl-substituent causes an increase in intensity of absorption at the shorter wavelength band (2400–2500 Å), but a 5-methoxyl group causes a decrease in intensity. These effects are reversed at the longer wavelength maxima, although the differences are less marked, and a distinct movement of the maxima towards shorter wavelength occurs when a 5-methoxyl group is present.

TABLE 1

OESTROGENIC ACTIVITY

| Compound | Total Dose per Mouse (μg) | Average Uterine Weight* | |
|---|---|---|---|
| | | Test (mg) | Control (mg) |
| 7,4′-Dimethoxy-4-phenyl*iso*flav-3-en . . . . | 11·7 | 4·5 | 2·3 |
| | 23·4 | 7·3 | ,, |
| | 46·7 | 10·4 | ,, |
| 7,4′ - Dimethoxy - 4 - (*p* - methoxyphenyl)*iso*-flav-3-en | 5·0 | 2·8 | 2·6 |
| | 26·0 | 6·8 | ,, |
| | 52·0 | 12·1 | ,, |
| 5,7,4′-Trimethoxy-4-phenyl*iso*flav-3-en . . | 322·0 | 3·2 | 2·4 |
| | 675·0 | 8·3 | ,, |
| | 1009·0 | 8·8 | ,, |
| 5,7,4′ - Trimethoxy - 4 - (*p* - methoxyphenyl)-*iso*flav-3-en | 442·0 | 4·2 | 2·7 |
| | 830·0 | 6·7 | ,, |
| | 1191·0 | 7·4 | ,, |

* Five mice.

The oestrogenic activities (Table 1) were determined by measuring the uterine weight increase in ovariectomized mice (Robinson 1949). 7,4′-Dimethoxy-4-phenyl*iso*flav-3-en and 7,4′-dimethoxy-4-(*p*-methoxyphenyl)-*iso*flav-3-en were both active at a total dose level of about 20 μg. A 5-methoxyl group (5,7,4′-trimethoxy-4-phenyl*iso*flav-3-en and 5,7,4′-trimethoxy-4-(*p*-methoxyphenyl)*iso*flav-3-en) reduces the activity 30-fold.

7,4′-Dimethoxy-4-phenyl- and 7,4′-dimethoxy-4-(*p*-methoxyphenyl)*iso*flav-3-en are more active than the corresponding 4-alkyl*iso*flav-3-ens (Bradbury and White loc. cit.), than triphenylethylene (300 μg) and triphenylchloroethylene (65 μg), and are within the range of activity of α-(4-hydroxyphenyl)stilbene (20 μg), 4,4′-dihydroxy-α-phenylstilbene (15 μg), and 4-methoxy-α-(*p*-methoxyphenyl)-β-bromostilbene (20 μg) quoted by Masson (1944).

### *Experimental*

All melting points are corrected. The ultraviolet absorption spectra were measured in ethanol solution by means of a Beckmann DU spectrophotometer. Microanalyses were carried out in the C.S.I.R.O. Microanalytical Laboratory.

(*a*) *7,4′-Dimethoxy-4-phenyl*iso*flav-3-en.*—7,4′-Dimethoxy*iso*flavanone (1 g) in dry benzene (20 ml) was added to a solution of phenylmagnesium bromide prepared from bromobenzene (1·5 g) and magnesium (0·17 g) in dry ether (20 ml). After refluxing for 16 hr, 10% aqueous hydrochloric acid (100 ml) was added with shaking, and the separated benzene-ether layer washed once with water and dried over calcium chloride. The residue obtained on evaporation of the solvent was heated under reduced pressure at 250 °C for 30 min, and on crystallization from ethanol gave a yellow solid (1·2 g), which when recrystallized from acetone formed colourless needles, m.p. 142 °C (Found: C, 80·1; H, 5·8; $CH_3O$, 17·1%. Calc. for $C_{23}H_{20}O_3$: C, 80·2; H, 5·85; $CH_3O$, 18·0%). Light absorption: $\varepsilon_{max.}$ 18,760 at 3250 Å.

(*b*) *7,4′-Dimethoxy-4-*(p*-methoxyphenyl*)iso*flav-3-en.*—The same quantities of reagents were used as in the last experiment but *p*-bromoanisole was substituted for bromobenzene. The product was an orange oil (1·8 g) from which an unidentified orange solid (0·11 g) separated from acetone. The mother liquors after stirring with ether and recrystallization of the solid obtained from ethanol gave colourless needles (73 mg), m.p. 141 °C (Found: C, 77·2; H, 5·95%. Calc. for $C_{24}H_{22}O_4$: C, 77·0; H, 5·9%). Light absorption: $\varepsilon_{max.}$ 20,450 at 2475 Å; 18,500 at 3250 Å.

(*c*) *5,7,4′-Trimethoxy-4-phenyl*iso*flav-3-en.*—When 5,7,4′-trimethoxy*iso*flavanone (1 g) was made to react with phenylmagnesium bromide according to the foregoing procedure the product (0·97 g) could be isolated without heating under reduced pressure. It formed colourless needles, m.p. 157 °C from ethanol (Found: C, 77·3; H, 6·0%. Calc. for $C_{24}H_{22}O_4$: C, 77·0; H, 5·9%). Light absorption: $\varepsilon_{max.}$ 19,150 at 3200 Å.

(*d*) *5,7,4′-Trimethoxy-4-*(p*-methoxyphenyl*)iso*flav-3-en.*—By the same procedure 5,7,4′-trimethoxy*iso*flavanone (1 g) and *p*-anisylmagnesium bromide gave a product crystallizing from ethanol as colourless needles (0·92 g), m.p. 136 °C (Found: C, 74·3; H, 6·0; $CH_3O$, 30·1%. Calc. for $C_{25}H_{24}O_5$: C, 74·2; H, 6·0; $CH_3O$, 30·7%). Light absorption: $\varepsilon_{max.}$ 18,910 at 3200 Å.

The author is indebted to Dr. D. E. White for advice during the course of this work, and to Dr. D. H. Curnow and Mr. A. B. Beck for biological testing.

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